A survey of the reproductive ecology and patterns of pollen-mediated gene flow in *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees

A thesis submitted in fulfilment of the requirements for the award of the degree
DOCTOR OF PHILOSOPY

By

Kym M. Ottewell, B. Sc. (Hons)
August 2007

UNIVERSITY OF ADELAIDE
School of Earth and Environmental Sciences

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Abstract

In many areas of south-eastern Australia, the clearance of temperate eucalypt woodlands for agriculture has been so extensive that only scattered remnant trees ("paddock trees") remain. The loss of habitat and increased spatial isolation of trees in paddocks is predicted to lead to a decline in plant fecundity because of disruptions to plant-pollinator interactions, which has important implications for the long-term persistence and maintenance of these populations. Paddock trees are currently in decline due to a range of threats and urgent action is required to address the chronic lack of recruitment to paddock tree populations to ensure their long-term viability. In order to assess the ability of paddock trees to contribute to population regeneration, I assessed the reproductive viability and patterns of mating of paddock trees of two woodland eucalypt species, *Eucalyptus camaldulensis* and *E. leucoxylon* in the Mt Lofty Ranges, South Australia. I examined the effects of spatial structure and pollination system (Bird vs. Insect) on the reproductive success of these two species. I predicted that *E. camaldulensis* paddock trees might suffer inadequate pollination services, and therefore lower fecundity, as a result of spatial dispersion of trees because of the lower mobility of insect-pollinators, as opposed to bird-pollinators.

My initial demographic survey found that populations of *E. camaldulensis* and *E. leucoxylon* paddock trees were heavily skewed towards larger and potentially older individuals than trees found in natural density vegetation, and that seedling and juvenile individuals were completely absent from the paddock tree environment. Nonetheless, adult paddock trees of both species contained high genetic diversity (in terms of observed heterozygosity and the number of alleles), demonstrating their importance as a reservoir of genetic variation. Despite the highly dispersed nature of these paddock tree populations (average tree density ~1 tree/ha, average distance to nearest conspecific ~70m), almost all trees flowered and set fruits. Not only were these trees reproductively viable, germination rates of seeds were comparable in paddock trees and trees in natural vegetation, indicating that paddock trees of both species were receiving adequate attention from pollinators.

Genetic analysis of progeny arrays confirmed that paddock trees of both species received significant outcrossed pollen from a diverse pool of donors. Overall,

population outcrossing rates for paddock trees were high (*E. camaldulensis*, 74% and *E. leucoxylon*, 82%) and did not differ significantly from trees in natural vegetation. Paternity analysis revealed extensive pollen dispersal amongst paddock trees of both species, with the distance of outcross pollen dispersal averaging ~300m for *E. camaldulensis* and ~520m for *E. leucoxylon*. In addition, I identified a number of mating events that occurred between *E. leucoxylon* paddock trees, and trees found in vegetation fragments, which highlights the role paddock trees may play in extending the pollination neighbourhoods of trees in small isolated patches.

Despite predictions that spatially isolated trees may be susceptible to reproductive decline due to the loss or alteration of plant-pollinator interactions, this study revealed that paddock tree populations of *E. camaldulensis* and *E. leucoxylon* were reproductively viable and received sufficient visits by pollinators that resulted in high outcrossing rates. In addition, *E. camaldulensis* did not appear to suffer reduced fecundity as predicted, perhaps due to the activities of the introduced European honeybee, *Apis mellifera*. The results of this study suggest that seed collected from these paddock trees are both genetically diverse and representative of adult populations; therefore, such paddock trees could contribute successfully to conservation strategies that sought to regenerate cleared paddocks. Furthermore, the genetic diversity present in the adults and the extensive network of pollen dispersal detected in this study suggests that paddock trees may play a vital role in maintaining connectivity in fragmented landscapes. However, ways to ensure that offspring recruit and persist in paddocks will need to be investigated to guarantee the long-term viability of these populations.

Declaration

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Kym Ottewell
August 2007

Acknowledgements

Chapter 1   General Introduction

1.1   Eucalypt woodlands

Eucalypts are widely distributed, long-lived and, by virtue of their height and biomass, are an obvious component of the ecosystems and landscapes in which they occur. They dominate the majority of vegetation associations throughout temperate Australia and grow in a wide range of habitats from semi-arid landscapes to wet forests and sub-alpine regions. The genus *Eucalyptus* is one of the most speciose genera of vascular plants in Australia, containing in the order of 800 species of forest, woodland and mallee trees (Brooker & Kleinig 2001).

Woodland eucalypt species are distinguished from forest and mallee trees primarily on the basis of tree size and canopy characteristics. Specht (1970) defines woodland trees as being single-stemmed and smaller (10-25m in height) than forest trees. As opposed to forest trees, the crowns of woodland trees generally occupy a major proportion of the tree height and have a projected canopy cover of between 5% and 30%. Prior to European settlement, eucalypt woodlands covered extensive areas of temperate southern Australia and Tasmania. In the south-east of the continent they formed a relatively continuous vegetation on the inland side of the Great Dividing Range, occurring from approximately 27°S in Southern Queensland to the lower south-east of South Australia, with a narrow strip running north and south of Adelaide (Moore 1970). Since settlement by Europeans, extensive clearance, fragmentation and degradation of Australia's native vegetation has taken place. Eucalypt woodlands occur over fertile soils and in areas of moderate to high rainfall, thus they gained the early attention of settlers and were rapidly cleared for cropland or towns, or were grazed and converted to exotic pasture (Beard & Sprenger 1984; Goldney & Bowie 1990; Prober *et al.* 1990; Bennett *et al.* 1994; Kirkpatrick & Gilfedder 1995). The conversion of temperate eucalypt woodlands to agricultural land represents one of the most significant vegetation changes in Australian history (Yates & Hobbs 1997). In total, it is estimated that approximately 500 000 km^2 of eucalypt woodland vegetation (of the original 1 012 047 km^2) had been cleared by the mid-1980's (AUSLIG 1990) and, unfortunately, native forests and woodlands have continued to be cleared at a rate of ~5000 km^2/year since that time (Beeton *et al.* 2006) .

1.1.1 Eucalypt woodland vegetation of South Australia and the Mount Lofty Ranges: Local context

In the state of South Australia, the area south of Goyder's line (which essentially follows the 250mm rainfall isohyet) is defined as being suitable for agriculture. Prior to European settlement, woodlands covered about 16% of this agricultural region, equivalent to 2.4 million hectares (Paton *et al.* 1999; Figure 1.1). Today, none of South Australia's temperate woodland systems are still intact, all having been cleared and fragmented by agriculture or urban development. In 1990, it was estimated that only 426 657ha of these woodlands remained (~17%), with only 117 316ha protected in any form of long term reserve system (Paton *et al.* 1999). The remaining 300 000ha of woodland vegetation primarily exists on private properties and includes patches of remnant vegetation that are currently grazed and also scattered trees over improved pasture.

Figure 1.1: Map of South Australia showing the location of Goyder's Line and the southern Mount Lofty Ranges bioregion.

The area to the south of Goyder's Line supports eucalypt woodland, forest and mallee vegetation, while to the north the area supports primarily chenopod shrubland and grasslands. Map source: Primary Industries and Resources, South Australia.

The Mt Lofty Ranges (MLR) is a band of hills that stretch ~200km from near the base of the Flinders Ranges in the north to the Fleurieu Peninsula in the south, skirting the city of Adelaide. It is one of the wettest areas of the state and originally supported

extensive eucalypt woodlands on the slopes and foothills, and open forests in the highest rainfall areas along the central spine. The proximity of the MLR to the settlement of Adelaide and the suitability of the region for agricultural development has meant that the MLR has been subject to intense clearing of vegetation and habitat modification, and this continues today, along with added pressure from urbanisation and a range of other land use activities (Figure 1.2).

In the MLR, eucalypt woodlands were disproportionately cleared as the lower elevation and shale-derived soils on which they occur were more amenable to agriculture (Paton *et al.* 1999). In some areas, only 2% of the pre-existing native vegetation remains while overall it is estimated that 93% of the native vegetation in the MLR has been removed (Robinson & Traill 1996; Paton *et al.* 1999). Similar to other regions throughout south-eastern Australia, the remaining vegetation of the MLR is highly fragmented and in some areas, could best be considered relictual (in the terminology of McIntyre & Hobbs 1999), comprising only scattered trees over exotic pasture.
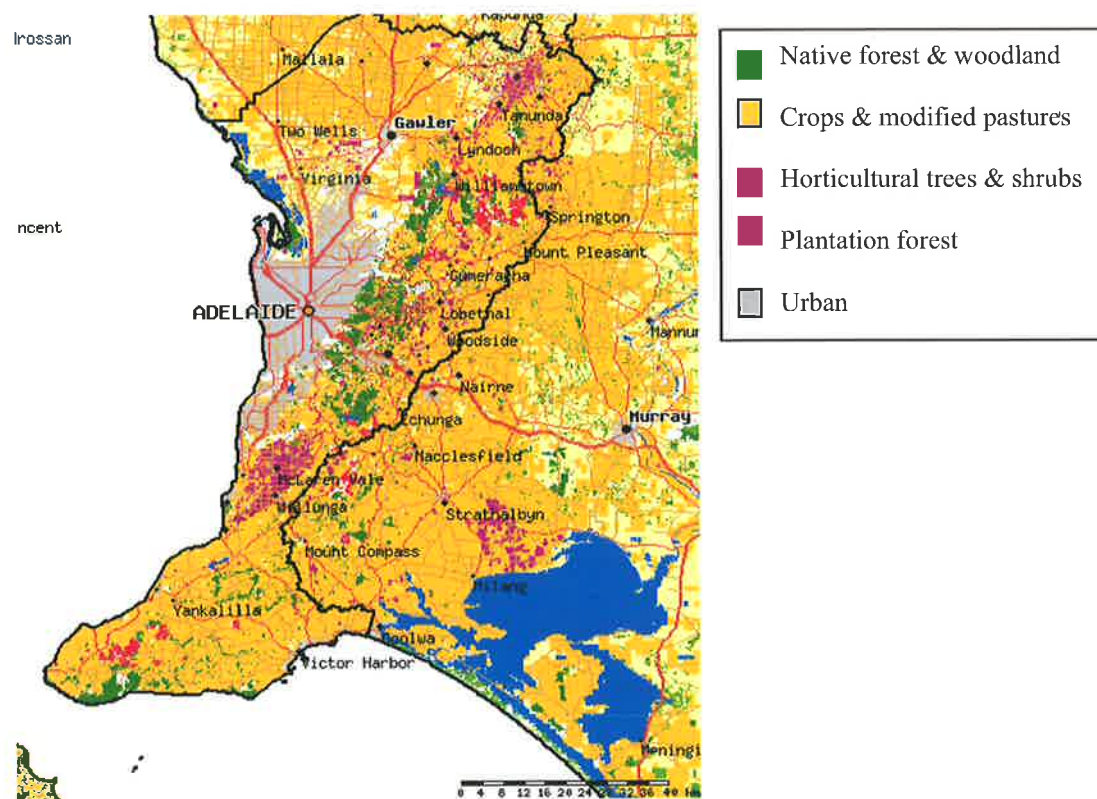
Figure 1.2: Current land use in the Southern Mount Lofty Ranges.

Source: Bureau of Rural Sciences, Integrated Vegetation Cover 2003, Version 1

The MLR have been identified as a National Biodiversity Hotspot[1] - an area of high conservation value in urgent need of action. The woodlands and forest vegetation of the MLR are isolated from other similar woodland systems in eastern Australia by more arid mallee habitats interspersed with chenopod shrublands. Consequently, the MLR contains many plant and animal species isolated from more extensive populations in the eastern states, and many of these species are at the western extremity of their distributional ranges (Paton *et al.* 2004b). Isolated populations are predicted to be less resilient to disturbance, and indeed the MLR has a dire record of species extinctions. Approximately 25% of native plant and animal species are currently listed as threatened taxa (Armstrong *et al.* 2003).

1.1.2 Native vegetation management in South Australia

South Australia leads the states in legislation protecting native vegetation from broad-scale clearing. The *Native Vegetation Management Act 1985* introduced controls over broad-scale clearing and provided for the establishment of Heritage Agreements, whereby native vegetation on private lands is reserved in perpetuity. Controls were further strengthened by the current *Native Vegetation Act 1991* and, currently, any application for the clearance of native vegetation must be assessed by the Native Vegetation Authority. However, the current Act still allows for the removal of scattered trees for farm management purposes, though greater awareness by the public and the Native Vegetation Council has appeared to have slowed the number of clearance applications granted approval in recent years (Native Vegetation Council Annual Report 2002). Nevertheless, Council approvals were granted for the removal of approximately 1000 mature trees in 2001-2 (Native Vegetation Council Annual Report 2002), with an estimated 50% of applications relating to woodland trees (Paton *et al.* 1999).

Across the agricultural landscapes of much of south-eastern Australia two types of remnant vegetation exists: (i) patches of more-or-less intact woodland and forest of varying size, quality and isolation (Prober & Brown 1994; Yates *et al.* 1994), and (ii) scattered trees (Ozolins *et al.* 2001; Gibbons & Boak 2002). Both vegetation types remain under ongoing ecological as well as anthropogenic pressures. Patches of remnant vegetation tend to be located mainly on rocky areas, upper slopes, poorer soils

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[1] http://www.pm.gov.au/news/media_releases/media_Release1084.html 20 Aug 2004

and floodplains subject to periodic inundation (Bennett 1993) - areas deemed unsuitable for agriculture but also less than optimal for native plant growth. While South Australia's *Native Vegetation Act 1991* protects remnant vegetation from broad scale clearing and provides for reservation of native vegetation in Heritage Agreements, the Act does not provide protection from a range of other threats to native vegetation. Remnant vegetation patches and scattered trees are further under threat from continuing clearance (legal and illegal), rising saline water tables and increased inundation, soil acidification, livestock grazing, nutrient enrichment, soil structural decline, altered fire regimes and the invasion of exotic weeds (Yates & Hobbs 1997; Paton *et al.* 1999). South Australian landholders, community groups and government agencies have attempted to address a number of the land degradation threats through significant revegetation efforts. However, all stakeholders agree that policies and facilities need to be put in place to better manage extant vegetation rather than focusing on revegetation (Dore *et al.* 1999; Moore & Renton 2002; 2003).

1.2   Scattered trees

Most of the scattered eucalypts in agricultural landscapes (also referred to as 'paddock' trees or 'isolated' trees) of the MLR were originally part of a woodland system, prior to the intervening vegetation being cleared. Extreme levels of habitat clearance mean that scattered trees typically exist at densities of less than 5% of the densities in the original woodland systems. Average densities of as little as 1 tree/ha now exist over extensive areas of south-eastern Australia (Sullivan & Venning 1982; Ozolins *et al.* 2001; Gibbons & Boak 2002; Carruthers *et al.* 2004). In a number of cases, scattered trees represent the last remnants of particular plant communities (Gibbons & Boak 2002; Carruthers *et al.* 2004).

From an ecological perspective, scattered trees play an important role in biodiversity conservation. Scattered trees have been shown to provide habitat for a wide range of animals that feed on nectar, pollen, seeds or invertebrates or that use hollows as nests. Many species of birds have been recorded using isolated trees (Lloyn & Middleton 1981; Fischer & Lindenmayer 2002a) including the endangered Regent Honeyeater, which nest and forage for nectar in remnant trees (Geering & French 1998) and the vulnerable superb parrot, which nests in hollows in paddock trees (Manning *et al.* 2004;

Manning *et al.* 2006).   In South Australian agricultural regions, 125 native bird species are known to use paddock trees (Carruthers *et al.* 2004) and Law *et al.* (2000) have observed 8 species of arboreal marsupials, 7 species of bats and 17 species of birds using paddock trees or patches of paddock trees in northern New South Wales. Scattered trees also support a diverse array of invertebrates including entire invertebrate food webs (Oliver *et al.* 2006), which provide food for reptile species in the paddock environment (Fischer *et al.* 2004).   While isolated trees provide habitat and a food resource for many animals, they also provide "staging posts" or "stepping stones" for animal movement across the landscape.   Koalas use isolated trees as they move between forested patches (Prevett 1991), as do many birds (Law *et al.* 2000; Fischer & Lindenmayer 2002b; Radford & Bennett 2006) and marsupial glider species (van der Ree *et al.* 2004).   The retention of paddock trees in grazed landscapes has also been shown to contribute to native plant conservation by maintaining indigenous herbs, forbs and mistletoes (Chilcott *et al.* 1997; Reid & Landsberg 1999) and they are an important source of seed for natural eucalypt regeneration (Dorrough & Moxham 2005).   The presence of scattered trees may allow a wide range of species to persist in even the most fragmented and degraded habitat systems and, on a landscape scale, paddock trees potentially provide connectivity between more intact blocks of remnant vegetation.

Paddock trees also have social and amenity value to landholders and the wider community. Paddock trees have many on-farm benefits including the provision of shade and shelter for livestock, a source of farm timber and fuelwood, and in some cases scattered trees may provide additional income to farmers through honey or essential oil production or as a source of seed for rural revegetation projects (Reid & Landsberg 1999). These trees also provide many ecosystem services. For example, soil under paddock trees remains more friable, has higher carbon, nitrogen and phosphorus concentrations and lower levels of soil acidification (Wilson 2002; Graham *et al.* 2004) and greater water infiltration rates (Eldridge & Freudenberger 2005) compared to the surrounding pasture environment, indicating the importance of scattered trees in conserving soil properties. Scattered trees generally have a large root volume and potentially intercept and transpire considerable volumes of subsurface water and nutrients thereby reducing land salinisation and stream eutrophication risks (Reid & Landsberg 1999; Eldridge & Freudenberger 2005). In addition, scattered trees are valued for their contribution to an overall landscape impression for both locals and

tourists, and their value for the promotion of regional tourism is high (Carruthers & Paton 2005).

Currently, many of the threats identified for patches of remnant vegetation also apply to paddock trees. As mentioned above, paddock trees continue to be legally cleared on agricultural properties, primarily for vineyards, irrigation and forestry. Some illegal clearance of native vegetation still continues with offenders rarely being successfully prosecuted (Paton *et al.* 1999). Surveys of tree size distribution in paddocks compared to trees in remnant vegetation (Carruthers *et al.* 2004; this thesis, Chapter 4), indicate paddocks contain predominantly older trees, most likely due to a lack of recruitment over the last 50-150 years. Thus, the rate of paddock tree loss is expected to increase dramatically over the next century due to natural senescence. Paddock trees also appear to suffer from disease (e.g. *Phytophthora*) and invertebrate attack more severely than trees in remnant vegetation (Sullivan & Venning 1982; Lowman & Heatwole 1992; Paton & Eldridge 1994), which has lead to high mortality rates of paddock trees in the south-east of South Australia (Paton *et al.* 1999).

The rate of paddock tree decline has been calculated for areas in the Lachlan Valley region in central New South Wales (Ozolins *et al.* 2001) and the south-east region of South Australia (Sullivan & Venning 1982). From aerial photographs, Ozolins *et al.* (2001) found that isolated trees declined in density from 0.37 trees/ha in the 1960's to 0.30 trees/ha in the 1990's, a decline of 20% over approximately 35 years. Similarly, Sullivan & Venning (1982) found that between 1945 and 1980, 8-64% of scattered trees had been lost from their survey sites (mean rate of paddock tree loss = 17.5% over 35 years). Such an alarming rate of tree loss is cause for concern in itself, considering the important role these trees play in the landscape and in biodiversity conservation. However, of even greater concern is the complete lack of recruitment in paddock tree ecosystems to replace the loss of existing trees. A broad scale survey of eucalypt establishment in agricultural landscapes in Victoria revealed eucalypt regeneration was virtually non-existent in areas with a current, frequent grazing regime (Dorrough & Moxham 2005). Some eucalypt regeneration has been observed in areas with light grazing or grazing followed by a long rest period, though the greatest amount of regeneration has been observed in areas protected from livestock grazing (Kirkpatrick *et al.* 2000; Dorrough & Moxham 2005). Lack of woodland eucalypt recruitment is also

of major concern throughout the majority of southern Australia (Petit *et al.* 1998; Yates *et al.* 2000; Manning *et al.* 2006).

There is clearly a great need for urgent action to be taken to halt the decline in isolated woodland trees and strategies need to be undertaken to increase seedling recruitment to populations. Revegetation projects in the MLR region have been relatively ad hoc to date, and, more often than not, have been established using non-local seed sources. Ideally, revegetation/restoration projects should consist of locally-sourced seed to preserve existing genetic variation and adaptations, and to reduce the risks of "genetic pollution" to surrounding vegetation (Wilkinson 2001; Hufford & Mazer 2003; Potts *et al.* 2003). Since scattered paddock trees are often the last remaining examples of the previous extant vegetation in any particular region, it would be beneficial if seed from these trees were to be used in revegetation projects or allowed to regenerate naturally (e.g. by fencing paddock trees to exclude grazing) to help maintain the genetic integrity of the woodland system. However, very little is known about the reproductive biology or ecology of paddock trees, and, in particular, the genetic quality of offspring produced by these trees. The fragmentation and thinning of vegetation has led to changes in the numbers, types and activities of native birds, mammals and invertebrates associated with remnant woodland vegetation (Ford & Bell 1981; Landsberg *et al.* 1990; Paton & Eldridge 1994; Paton *et al.* 2004b); consequently biotic processes such as plant-pollinator interactions have suffered serious perturbation (Olesen & Jain 1994; Yates & Hobbs 1997; Paton *et al.* 2004b). Very little data exists on the reproductive "health" of scattered trees. Are pollinators still active in such a highly modified system? Are the required pollinator species still present? Has the increased spatial isolation of trees altered pollinator behaviour? What are the consequences for plant mating patterns and, ultimately, seed quality?

1.3 This study

In this thesis I investigate the demography and reproductive dynamics of remnant scattered tree populations of the woodland eucalypt species, *Eucalyptus camaldulensis* and *E. leucoxylon*. Both species have broadly similar growth habits and are members of the same subgenus of *Eucalyptus* (*Symphyomyrtus*) though belong in different taxonomic sections of the genus (*E. camaldulensis*, Section Exsertaria; *E. leucoxylon,*

Section Adnataria). Both *E. camaldulensis* and *E. leucoxylon* are widespread throughout the southern MLR and frequently co-occur in the same vegetation communities. Each species has high moisture and soil nutrient requirements and thus, most typically occur in areas of the MLR deemed most suitable for agriculture. Consequently, both species have been extensively cleared in agricultural areas of the MLR. In natural populations *E. camaldulensis* and *E. leucoxylon* may occur at densities of up to 100 trees/ha, however, as scattered trees, experience densities as low as 1 tree/ha across much of their distribution in the MLR.

The majority of studies of the effects of habitat fragmentation on plant populations have recorded negative impacts on plant reproduction; for example, reduced fruit or seed set, reduced outcrossing, reduced seed viability (reviewed in the following chapters). These effects may arise due to altered plant mating patterns as a result of the loss of pollinator species, changes in pollinator behaviour in response to fragmentation and changes in the number of available or compatible mating partners, amongst other perturbations (reviewed in Hobbs & Yates 2003; Ghazoul 2005; Aguilar *et al.* 2006). Reduced reproductive output of fragmented plant populations has important implications for the continued survival and viability of remnant populations. In addition, alterations to plant mating patterns due to habitat fragmentation may lead to the erosion of genetic variation in remaining plant populations and lead to an increased risk of extinction through increased inbreeding, reduced inter-population gene flow and increased genetic drift (Young *et al.* 1996).

To date, the majority of the habitat fragmentation literature has focused on remnant patches of vegetation where local plant densities are more or less the same as what they were pre-fragmentation. It is only recently that researchers have turned their attention to the scattered remnant trees that are left after tree thinning and the conversion of a diverse natural habitat to pastoral habitat (e.g. Chase *et al.* 1996; Dick 2001; Cascante *et al.* 2002). One of the major predictions of the impact of increased spatial isolation of fragments is that plants in isolated population fragments may receive fewer pollinator visits and therefore reduced opportunities for cross-pollination and outbreeding, leading to reproductive decline (Hobbs & Yates 2003). However, the severity of the impacts of spatial isolation on plant reproduction are likely to be determined by a range of factors including the degree of specialisation of plant-pollinator interactions (e.g. reliance on

specialist pollinators vs. generalist pollinators) and pollinator characteristics (e.g. foraging behaviour, flight capabilities), as well as the mating system of the plant species in question (e.g. self-compatible vs. self-incompatible) (Ghazoul 2005; Aguilar *et al.* 2006).

Like most eucalypts, both *E. camaldulensis* and *E. leucoxylon* have a mixed mating system, with predominant outcrossing but they are also capable of selfing (Ellis & Sedgley 1993; Moncur *et al.* 1995). All *Eucalyptus* species produce nectar as an attractant and floral reward for pollinators, however, very few species exhibit specialisations for any particular pollination syndrome and most are visited by a diverse array of invertebrates (e.g. native bees, flies, wasps, ants, beetles) and, in some cases, also by a range of vertebrates (e.g. possums, honeyeaters, lorikeets) (House 1997). *Eucalyptus camaldulensis* produces numerous, small, cream-colored flowers and is pollinated predominantly by insects, though birds have also occasionally been observed foraging on *E. camaldulensis* flowers (Paton, pers. comm.). In contrast, *E. leucoxylon* produces fewer and larger flowers than *E. camaldulensis*, which are cream to red-colored and are pollinated predominantly by birds (Paton & Ford 1977), though invertebrate floral visitors are also common. In addition to native pollinators, both tree species are visited by introduced honeybees (*Apis mellifera*), which are common in the agricultural environment.

In this study, I aimed to contrast the effects of increased spatial dispersion (reduced tree density, increased distance between individuals) of eucalypt populations on the reproductive output of trees with predominantly insect pollination and predominantly bird pollination. It is generally observed that bird pollinators are capable of making movements over distances of several hundreds of metres to kilometres, whereas insect pollinators may only make movements of several metres to tens of metres (Paton 1996; Paton 2000; Paton *et al.* 2004b). Indeed, several studies have shown that insect-pollinated eucalypt species may be effectively reproductively isolated over distances of 100-250m (Prober & Brown 1994; Butcher *et al.* 2005), commonly the distance separating paddock trees in the MLR. I therefore predict that *E. leucoxylon* may be less susceptible to reproductive decline as a result of increased spatial dispersion of adult trees than *E. camaldulensis*, since, due to their greater flight capabilities, bird pollinators may be more likely than insect pollinators to maintain pollination services

over the range of isolation distances experienced by *E. leucoxylon* and *E. camaldulensis* paddock trees. Specifically, I predict that insect-pollinated *E. camaldulensis* trees may experience:

- reduced fecundity, manifested as reduced fruit and/or seed production, as a result of limited pollinator visitation and/or increased seed abortion due to incompatible matings (e.g. selfing)

- increased geitonogamous selfing, manifested as a higher proportion of selfed offspring in progeny arrays, as a result of increased foraging bout length of pollinators in accordance with optimal foraging theory

- reduced number of males contributing to the seed crop, manifested as a higher correlation of paternity within progeny arrays, as a result of the lower mobility of insect pollinators

- smaller scale of effective pollen dispersal due to the limited mobility of insect pollinators

In this thesis I firstly explore the demographic and genetic structure of remnant tree populations of *E. camaldulensis* and *E. leucoxylon* at two locations in the MLR. I then survey reproductive patterns in each population, initially to evaluate the reproductive status of paddock trees of each species, and to investigate some of the potential determinants of reproductive success. Finally, I use genetic markers to further explore the reproductive dynamics of paddock tree populations by measuring aspects of the effective mating system, including estimating individual outcrossing rates, the number of individuals contributing to reproduction and the spatial distance of effective pollen dispersal.

Scattered trees occur in many human-modified habitats across the globe (Manning *et al.* 2006). While this study will provide insights into the reproductive dynamics of scattered eucalypt tree populations in an Australian context, it is hoped that this study will also contribute to the growing body of knowledge and understanding of the ecology of this particular type of remnant vegetation and will lead to better management and protection of scattered trees both in Australia and overseas. In particular, by using a molecular genetic approach, I will be able to provide a "real-time" estimate of the patterns of pollen dispersal in spatially isolated trees and, thus, an assessment of the

genetic quality of seeds collected from these trees and their potential contribution to long-term species conservation. Information from this study will enable land managers in south-eastern Australia to better manage extant populations of paddock trees and to guide restoration efforts to address the chronic lack of recruitment in these populations.

Chapter 2   Genetic markers for population genetic analyses and mating system estimation in *Eucalyptus camaldulensis* and *E. leucoxylon*

Note: The proportion of this chapter describing the isolation of microsatellite markers for *Eucalyptus leucoxylon* has been published in the Journal of Heredity:

K. M. Ottewell, S. C. Donnellan, G. F. Moran and D. C. Paton (2005). Multiplexed microsatellite markers for the genetic analysis of *Eucalyptus leucoxylon* (Myrtaceae) and their utility for ecological and breeding studies in other *Eucalyptus* species. Journal of Heredity 96(4): 445-451.

2.1   Introduction

2.1.1   Microsatellite sequence evolution

Repetitive DNA sequences of different types occur frequently in eukaryotic genomes (Charlesworth *et al.* 1994) and can make up the major fraction of most plant nuclear genomes (Kubis *et al.* 1998). One particular class of repetitive DNA are microsatellites, which are tandem repeats of short DNA segments, typically one to six bases in length. They evolve rapidly and have been shown to be highly polymorphic for repeat number. Copy numbers of individual repetitive DNA motifs can vary from a few to more than 70 (Brohede *et al.* 2002; Beck *et al.* 2003), though repeats of more than 30 units are relatively rare (Kruglyak *et al.* 2000; Ellegren 2002). Microsatellites constitute a large fraction of non-coding DNA in most genomes and are relatively rare in protein-coding regions (Wang *et al.* 1994). Microsatellites themselves are non-coding but they may have many functional effects in gene expression, genetic disorders, chromatin organisation and cell cycle and DNA metabolic processes (reviewed in Li *et al.* 2002). On the whole, microsatellites are expected to be selectively neutral (Awadalla & Ritland 1997; Schlotterer & Wiehe 1999). However, natural selection may act on patterns of microsatellite diversity indirectly when the microsatellite has functional significance (Li *et al.* 2000a; Li *et al.* 2000b) or when the microsatellite is in close linkage to a selected locus (genetic "hitchhiking", Slatkin 1995a).

In vitro experiments suggest that replication slippage is the main mechanism responsible for the formation and expansion of microsatellite arrays (Schlotterer &

Tautz 1992), most commonly resulting in the addition or deletion of single repeat units. The alternative model, that of unequal crossing-over or gene conversion during meiosis or mitosis (Hancock 1999), can potentially lead to allele length variants by the addition or deletion of a number of repeat units but appears to be less common (Jarne & Lagoda 1996).

The mutation rate at microsatellite loci is particularly high in comparison to other molecular markers (e.g. microsatellite mutation rates are 10^6-10^7 times higher than the substitution rates at nuclear genes of plants (Wolfe *et al.* 1987; Hancock 1999), but has been shown to vary greatly between taxa, between loci and even amongst alleles at a locus. Estimates of microsatellite mutation rates range from 10^{-3} to 10^{-4} in humans (Weber & Wong 1993), 10^{-3} to 10^{-5} in mice (Dallas 1992), ~10^{-6} in *Drosophila melanogaster* (Schlotterer *et al.* 1998), 10^{-2} to 10^{-3} in chickpea (Udupa & Baum 2001), ~10^{-5} in maize (Vigouroux *et al.* 2002) and 10^{-4} in durum wheat (Thuillet *et al.* 2002). The rate of slippage during replication of simple DNA sequences has also been shown to be dependent on the nature of the repeat sequence, for example, whether it is di-, tri- or tetranucleotide (Schlotterer & Tautz 1992; Chakraborty *et al.* 1997; Schlotterer *et al.* 1998), with some studies showing that AT-rich microsatellites have higher slippage rates than other motifs (Kruglyak *et al.* 2000; Udupa & Baum 2001). Mutation rate also varies with allele length; slippage is more frequent with greater numbers of tandem repeats (Wierdl *et al.* 1997; Brohede *et al.* 2002; Beck *et al.* 2003). Other kinds of mutations (e.g. point mutations) in repeat regions that interrupt stretches of perfect repeats decrease the frequency of slippage and may act to limit the size of microsatellite regions (Jin *et al.* 1996; Kruglyak *et al.* 2000; Santibanez-Koref *et al.* 2001).

2.1.2   The utility of microsatellite markers in genetic studies

Identified in the 1980's, microsatellites have rapidly become the marker of choice for population genetic investigations because their polymorphic nature and rapid evolutionary rate makes them extremely sensitive to changes in population breeding size, structure and rates of dispersal (Slatkin 1995b). In addition, they are easily assayed in the laboratory. Microsatellites are assayed by polymerase chain reaction (PCR) amplification by using primers designed to match unique DNA sequence flanking the microsatellite region, and length variation is determined by electrophoresis

on standard or specialised (e.g. automated sequencer) laboratory equipment. Microsatellite alleles can be scored with relative ease and reliability, in comparison to other population genetic markers (e.g. allozymes, RAPDs), based on their size. Also, microsatellites are inherited co-dominantly. That is, at microsatellite loci heterozygotes can be distinguished from homozygotes, and therefore microsatellites can be far more informative than dominant markers such as RAPDs and AFLPs.

Increasingly, evolutionary and ecological studies are benefiting from the analysis of individual-based genotypic information. Parentage-type studies involve the assessment of precise parental relationships within populations and have lead to insights into the social structure, mating patterns, kinship and quantification of reproductive success of many plant and animal populations (e.g. Paetkau *et al.* 1995; Dow & Ashley 1998a; Dow & Ashley 1998b). Microsatellites are ideal for parentage studies because they have many relatively rare alleles per locus. By sampling multiple microsatellite loci it is easy to assemble unique genetic "fingerprints" of all individuals in a population, and therefore it is possible for researchers to follow the movement of genes (for example, via pollen or seed) across the landscape. The only assumptions required for microsatellite paternity analysis are that there are no mutations between parents and offspring and that adults and offspring with matching genotypes are related. The mutation rate of microsatellite sequences (between 10^{-2} to 10^{-5} mutations per locus per generation) is low enough that the probability of a mutation between parents and offspring is negligible when small numbers of offspring are sampled.

False parentage assignments may also occur when two candidate parents share the same genotype, by kinship or by chance. The use of microsatellite markers greatly increases our ability to precisely characterise individuals genetically due to the high allelic variation present at microsatellite loci. The ability to discriminate between individuals based on their multi-locus genotypes is essentially a function of the number of loci sampled and the allelic diversity at those loci. The power of a panel of microsatellites to precisely identify individuals can be calculated using the "probability of identity" measure (P_{ID}) (Paetkau *et al.* 1995) and the ability to exclude individuals in parentage assignment studies can also be calculated (the "probability of exclusion", Weir 1998). In general, the greater the number of microsatellite loci sampled the higher the resolving

power of the loci, although a smaller number of loci are required when allelic diversity per locus is high (Bernatchez & Duchesne 2000).

A number of statistical methods are currently available to assign parentage in paternity and gene flow studies, based on simple exclusion, likelihood methods or categorical or fractional parentage assignment (Jones & Ardren 2003). However, a number of problems may be encountered when attempting parentage assignment based on microsatellite genotypes. The most common is the non-amplification of certain alleles ("null alleles") that can occur due to substitutions, insertions or deletions within the priming sites (e.g. Paetkau *et al.* 1995). The presence of null alleles may cause problems with apparent parent-offspring mismatches in paternity assignment studies. Potentially, another source of error may occur due to somatic mutations at microsatellite loci that occur during mitotic division. Very little is known about the frequency of somatic mutations at hypervariable loci, which also have the potential to create apparent parent-offspring mismatches during mating system and parentage analyses. The issue of somatic mutations at microsatellite loci will be addressed in Chapter 3.

2.1.3   Selecting microsatellite markers for *E. camaldulensis* and *E. leucoxylon*

The development of microsatellite markers for population genetic assays can be a costly and time-consuming exercise (Zane *et al.* 2002; Squirrell *et al.* 2003). Alternatively, a number of studies have shown that primers developed for microsatellite loci in one species can be successfully transferred to congeneric species, and in some cases microsatellite primers have been successfully transferred across families (e.g. birds, Primmer *et al.* 1996). However, the risk of mutations in primer, flanking and microsatellite sequences generally increases with the evolutionary distance among taxa, increasing the risk of null alleles (Primmer *et al.* 1996; Peakall *et al.* 1998). A number of microsatellite loci have been isolated from *Eucalyptus* species and primer sequences have been published in the scientific literature. These include four loci isolated from *E. nitens* (Byrne *et al.* 1996); 70 loci from *E. grandis* and *E. urophylla* (Brondani *et al.* 1998; Brondani *et al.* 2002) and 8 loci from *E. sieberi* (Glaubitz *et al.* 2001). Byrne *et al.* (1996) reported 100% conservation of *E. nitens* microsatellite primers to three other species within the subgenus *Symphyomyrtus* and Brondani *et al.* (2002) reported 95% transferability of their *E. grandis/E. urophylla* primers to two other Symphyomyrt

species. Fourteen microsatellite loci have also been isolated from the closely-related genus *Corymbia* (*Corymbia variegata*, Jones *et al.* 2001), of which, 53% of loci transferred to *E. nitens* and *E. globulus*, two *Symphyomyrtus* species. Thus, it appears that there are relatively high levels of microsatellite conservation within the *Eucalyptus* subgenus *Symphyomyrtus*.

Additional microsatellite markers, isolated from *E. globulus*, an important forestry species, are available from CSIRO Forestry and Forestry Products located in Canberra, Australia (G. Moran, unpubl. data). Since the *E. globulus* microsatellite markers have been well characterised by CSIRO, these were determined to be the most appropriate to be tested for transferability to my two study species, *E. camaldulensis* and *E. leucoxylon*. *Eucalyptus camaldulensis*, *E. leucoxylon* and *E. globulus* all fall within the largest eucalypt subgenus *Symphyomyrtus*. However, the three species belong to different taxonomic Sections within *Symphyomyrtus* – *E. camaldulensis* belongs to *Exsertaria*, *E. leucoxylon* belongs to *Adnataria* and *E. globulus* belongs to *Maidenaria*. Unlike the other microsatellite markers described above, the microsatellites isolated from *E. globulus* are trinucleotide repeats, which are easier to score than the more commonly isolated dinucleotide microsatellites due to a reduction in the frequency of stuttering that occurs during the PCR process (Scotti *et al.* 2002).

2.1.4 Aims

In this chapter I describe the process involved in selecting microsatellite loci for use in population genetic and parentage studies in *Eucalyptus leucoxylon* and *E. camaldulensis* trees. I have divided the chapter into three, relatively self-contained sections. In the first section, *E. globulus* microsatellite primers were screened across individuals of both species to determine whether a suitable number of microsatellite loci for population genetic analyses could be transferred to each species. An appropriate number of *E. globulus* microsatellite primers were successfully transferred to *E. camaldulensis*, which were then assessed for polymorphism and allelic diversity in the *E. camaldulensis* study population at Tungkillo, South Australia. An insufficient number of microsatellite primers transferred to *E. leucoxylon*; consequently, the third section of this chapter describes the process used to isolate microsatellite loci specifically for *E. leucoxylon*. Genetic diversity measures for *E. leucoxylon* microsatellite markers were assessed in

two study populations (Flaxley and Ngarkat Conservation Park, South Australia). In addition, the degree to which microsatellite primer sequences are conserved amongst eucalypt species was assessed using *E. leucoxylon* microsatellite primers tested in a taxonomically diverse range of eucalypt species and two closely related genera.

2.2 Transferability of *Eucalyptus globulus* microsatellite primers to *E. camaldulensis* and *E. leucoxylon*.

In order to assess the suitability of *E. globulus* microsatellite primers for use in *E. camaldulensis* and *E. leucoxylon* species, the following screening protocol was observed:

- Firstly, I tested whether microsatellite primers developed for *E. globulus* amplified PCR products in *E. camaldulensis* and *E. leucoxylon,* and whether the PCR products were of similar size to those identified for *E. globulus*. This provided initial evidence that the primers were amplifying the same microsatellite locus in each species;

- I tested whether microsatellite loci in *E. camaldulensis* and *E. leucoxylon* were polymorphic; that is, whether a range of allele sizes was observed in the DNA samples tested;

- Lastly I assessed whether allelic patterns were consistent with that of the target microsatellite based on a model of step-wise mutation. I made the assumption that a step-wise pattern of allele sizes would be suitable evidence to confirm the presence of a microsatellite. It was not necessary to sequence the putative microsatellite locus to confirm the structure of the microsatellite as this study involves only intraspecific comparisons and analyses do not rely on the evolutionary history of alleles.

Using these criteria, I aimed to identify a panel of 6-10 microsatellite loci that amplified consistently in the two target species and that displayed high levels of polymorphism, making them suitable for mating system and parentage analyses in *E. camaldulensis* and *E. leucoxylon*.

2.2.1   Sampling and DNA methods

Eucalyptus globulus microsatellite primers provided by CSIRO FFP were screened in ten individuals of each species, *E. camaldulensis* and *E. leucoxylon*. It was necessary to screen the microsatellite primers in a range of individuals, first to determine whether the target microsatellite loci were present in all individuals across the study region in South Australia and, second, to assess the degree of polymorphism at each microsatellite locus. Leaf samples were collected from individuals of each species located in the Adelaide metropolitan area and the MLR, as well as additional samples from the Waite Arboretum (**Table 2.1**). Leaf samples (typically 4-5 healthy leaves) were removed from trees, placed into plastic zip-lock bags in a cool, dry esky and transferred to a laboratory refrigerator or freezer at the end of the day. Genomic DNA was extracted from 3-5g of fresh or frozen (-20°) leaf material using a modified CTAB method described in Byrne *et al.* (1993) and visualised on a 1.5% agarose gel stained with Ethidium Bromide.

Thirteen *Eucalyptus globulus* primer pairs were tested on *E. camaldulensis* and *E. leucoxylon* DNA samples. In addition, one primer pair described in Brondani *et al.* (1998) was also tested since it had proved variable in *E. globulus*. Details of primer names, microsatellite motif and allele size ranges are provided in Table 2.2. *Eucalyptus globulus* primer sequences are available from the CSIRO FFP web page at http://www.ffp.csiro.au/tigr/molecular/eucmsps.html. PCR reactions were performed in a 10μl reaction volume containing the following reagents: 1x Promega PCR buffer, 250μM each dNTP, 1.5-2mM $MgCl_2$, 0.16 - 2μM each primer (F/R), 0.04U Promega Taq polymerase and approximately 10-20ng template DNA. PCR reactions were performed on a Corbett thermal cycler with the following cycling conditions: 95°C for 2 mins (1 cycle); 94°C for 10 sec, 52°C for 30 sec, and 72°C for 30 sec for 30 cycles; and 72°C for 30 mins (1 cycle). The *E. globulus* microsatellite primers were fluorescently-labelled at the 5' end with a FAM, TET or HEX. This allowed PCR products to be visualized on an ABI-Prism 310 automated sequencer. PCR products were sized using the Genescan and Genotyper software (Applied Biosystems) in reference to an internal size standard (TAMRA500, Applied Biosystems).

Table 2.1: List of *E. camaldulensis* and *E. leucoxylon* samples in which *E. globulus* microsatellite primers were tested.

DNA ID	Species	Location	Region[a]
EC01	*E. camaldulensis*	River Torrens, Hackney	AP
EC02	*E. camaldulensis*	Tree 844855, Botanic Park, North Adelaide	AP
EC03	*E. camaldulensis*	Blackwood	AP
EC04	*E. camaldulensis*	Jupiter Creek Gold Diggings, Echunga	MLR
EC05	*E. camaldulensis*	Meadows	MLR
EC06	*E. camaldulensis*	Tungkillo	MLR
EC07	*E. camaldulensis*	North Adelaide	AP
EC08	*E. camaldulensis*	River Torrens, North Adelaide	AP
EC09	*E. camaldulensis*	Tree 2212, Waite Arboretum	AP
EC10	*E. camaldulensis*	Tree 1529, Waite Arboretum	AP
EL01	*E. leucoxylon*	Tree 116, River Torrens, Hackney	AP
EL02	*E. leucoxylon*	Belair National Park	MLR
EL03	*E. leucoxylon*	Tree 21, Flinders University Flora Park	MLR
EL04	*E. leucoxylon*	Mt Bold Reservoir, Scott Creek	MLR
EL05	*E. leucoxylon*	Saddlebags Rd, Kangarilla	MLR
EL06	*E. leucoxylon*	Range Rd West, Willunga	MLR
EL07	*E. leucoxylon*	Wicks Rd, Wickham's Hill	MLR
EL08	*E. leucoxylon*	Pococks Rd, Echunga	MLR
EL09	*E. leucoxylon*	Tree 1615, Waite Arboretum	AP
EL10	*E. leucoxylon*	Tungkillo	MLR

[a] AP = Adelaide Plains, MLR = Mount Lofty Ranges

Table 2.2: Details of *E. globulus* and *Embra* microsatellite primers tested in *E. camaldulensis* and *E. leucoxylon*.

Locus	Motif	Fluorescent label	Size range in *E. globulus* (bp)[a]
Eg67	$(CTT)_8(CT)_7(TC)_5$	Hex	160-184
Eg30	$(CTT)_{20}$	Fam	290-340
Eg86	$(CTT)_{29}$	Tet	196-264
Eg84	$(CT)_7(CTT)_7$	Tet	118-138
Eg96	$(GAG)_8$ & $(GAA)_5$	Fam	276-288
Eg99	$(CTT)_{11}$	Fam	189-198
Embra2	$(AG)_{15}$	Hex	126-138
Eg16	$(GA)_2(TGA)_7$	Tet	235-242
Eg98	$(CTT)_9$	Tet	164-180
Eg126	$(GAA)_8$	Fam	332-351
Eg76	$(CTT)_{17}$	Hex	127-153
Eg117	$(CTT)_{13}$	Tet	168-186
Eg91	$(GAA)_6$	Fam	149
Eg65	$(ATG)_{12}$	Hex	252

[a] Allele size ranges provided by CSIRO Forestry and Forest Products, Canberra

2.2.2   Transferability of *Eucalyptus globulus* microsatellite primers to *E. camaldulensis* and *E. leucoxylon*

Of the fourteen *E. globulus* microsatellite primers trialed in *E. camaldulensis* and *E. leucoxylon*, eleven primer pairs amplified a product of approximately the expected size in *E. camaldulensis* (Table 2.3a), but only seven primer pairs amplified a product in *E. leucoxylon* (Table 2.3b). Of the eleven primer pairs that amplified in *E. camaldulensis*, three primer pairs were further discounted from use due to lack of polymorphism (*Eg126*) or inconsistent banding patterns (*Eg86* appeared to amplify two loci, while *Embra2* amplified products ~20bp smaller than that detected in *E. globulus* (Table 2.3a). Similarly, of the seven primer pairs that amplified a product in *E. leucoxylon*, two primer pairs were discounted because they were monomorphic or had low polymorphism (*Eg126, Eg87*), and *Eg67* was discounted due to the possibility of null alleles (Table 2.3b). Initial observations of allele sizes for the remaining polymorphic loci (*Eg67, Eg84, Eg96, Eg99, Eg16, Eg98, Eg91, Eg65* for *E. camaldulensis; Eg84, Eg96, Eg91, Eg65* for *E. leucoxylon*) indicated that variation in size was consistent with a pattern of stepwise mutation expected of microsatellite loci (e.g. Jarne & Lagoda 1996).

The desirable outcome of this screening trial was to obtain primers that amplified 6 – 10 microsatellite loci in each species. This was achieved in *E. camaldulensis* with eight (putative) microsatellite loci displaying consistent banding patterns and sufficient polymorphism to warrant further characterisation (see Section 2.3). Unfortunately, only four *E. globulus* primer pairs amplified in *E. leucoxylon*, an insufficient number for the type of analyses to be conducted in this project. There was potential to trial other microsatellite primers from other sources (e.g. those developed for *E. sieberi* (Glaubitz *et al.* 2001) or *E. grandis* and *E. urophylla* (Brondani *et al.* 1998)) in *E. leucoxylon*. However, these primers were developed in taxonomically more distant species to *E. leucoxylon* than *E. globulus* and therefore would have been even less likely to amplify in *E. leucoxylon*. Thus, it was necessary to isolate microsatellite markers specifically from *E. leucoxylon*. This process is described later in this chapter (section 2.4).

Table 2.3: Transferability of *E. globulus* microsatellite primers to *E. camaldulensis* and *E. leucoxylon*.

a. *E. camaldulensis*

Locus	Present in *E. camaldulensis*[a]	Size Range (bp)[b]	No. of alleles[b]	Comments
Eg67	Yes	153-185	7	
Eg30	No	-	-	
Eg86	Yes	160-240	6	Two loci?
Eg84	Yes	100-128	9	
Eg96	Yes	272-288	6	
Eg99	Yes	181-197	6	
Embra2	Yes	106-112	4	Size range inconsistent
Eg16	Yes	232-242	4	
Eg98	Yes	168-178	4	
Eg126	Yes	326	1	Monomorphic
Eg76	No	-	-	
Eg117	No	-	-	
Eg91	Yes	134-148	6	
Eg65	Yes	233-247	4	

b. *E. leucoxylon*

Locus	Present in *E. leucoxylon*[a]	Size Range (bp)[b]	No. of alleles[b]	Comments
Eg67	Yes	175-215	5	Null alleles?
Eg30	No	-	-	
Eg86	Yes	278-286	2	
Eg84	Yes	95-113	6	
Eg96	Yes	270-302	7	
Eg99	No	-	-	
Embra2	No	-	-	
Eg16	No	-	-	
Eg98	No	-	-	
Eg126	Yes	326	1	Monomorphic
Eg76	No	-	-	
Eg117	No	-	-	
Eg91	Yes	130-154	4	
Eg65	Yes	247-257	5	

[a] Whether primers amplified a product of approximately the same size in *E. camaldulensis* or *E. leucoxylon* as in *E. globulus*. [b] Allele size range and number of alleles observed in ten *E. camaldulensis* or ten *E. leucoxylon* individuals.

Several authors have reported high levels of microsatellite primer conservation amongst the *Eucalyptus* subgenus *Symphyomyrtus* (Byrne *et al.* 1996; Brondani *et al.* 1998). In this study, 80% (11/14) of *E. globulus* microsatellite primers successfully amplified a product in *E. camaldulensis,* but, of these, only eight were useable (8/14 = 57%). 50% (7/14) of *E. globulus* microsatellite primers successfully amplified a product in *E. leucoxylon,* and only 29% (4/14) were considered useable. Thus, whilst high rates of transfer of microsatellite primers have been reported amongst eucalypt species, the number of *useable* primers can be significantly less.

2.3   Characterisation of microsatellite markers for *Eucalyptus camaldulensis*

I further characterised *E. globulus* microsatellite markers in *E. camaldulensis* by screening them in adult trees from my study population at Tungkillo, South Australia. In this section, I genotyped 63 *E. camaldulensis* individuals at the eight selected microsatellite loci to better assess these markers for levels of polymorphism and their suitability for population genetic and parentage analyses in the study population.

2.3.1   Sampling and DNA methods

Scattered *E. camaldulensis* trees were located on agricultural properties surrounding the township of Tungkillo, South Australia (323700E/6144400N). Detailed tree and site descriptions are provided in Chapter 4. Leaf material was collected from the lower canopy of adult trees for genetic analysis as described previously.

Genomic DNA was extracted from 3-5g of fresh or frozen (-20°C) leaf material using the method described in Byrne *et al.* (1993). To aid in rapid throughput for genetic screening of individuals, the microsatellite primers were PCR multiplexed (i.e. 2-3 loci amplified in the same PCR reaction). During the testing phase, primer concentrations in each multiplex were optimised (Table 2.4) so that they amplified products of approximately equal intensity when visualised on an automated sequencer. All reactions were performed in a 15µl volume which contained: 1x AmpliTaq Gold PCR Buffer, 2mM MgCl$_2$, 0.2mM each dNTP, primer pairs as described in Table 2.4, 0.03U AmpliTaq Gold polymerase and ~10-20ng DNA. PCR reactions were performed on either a Hybaid OMNE-200 or an Eppendorf Mastercycler thermal cycler. The

following touch-down cycling program was used: 94°C for 9 min (1 cycle); 94°C for 30sec, 65-55°C (step down 2°C for each cycle) for 30sec, 72°C for 45sec (10 cycles); 94°C for 30sec, 55°C for 30sec, 72°C for 45sec (20 cycles); final extension 72°C for 12min.

Eucalyptus globulus microsatellite primers with overlapping allele size ranges were labelled with different fluorescent dyes (either FAM, TET or HEX) and primers with non-overlapping allele size ranges were labelled with the same dye. In this way, all eight loci could be electrophoresed in a single lane on an ABI-Prism 377 automated sequencer, reducing the cost of fluorescent genotyping. Fluorescently-labelled PCR products were electrophoresed on a 5% denaturing gel (Long Ranger, 6M Urea/1xTBE) and product sizes determined by comparison to an internal marker (TAMRA500, Applied Biosystems) using the program Genotyper (Applied Biosystems).

Table 2.4: *Eucalyptus camaldulensis* **microsatellite PCR multiplexes.**

Details of microsatellite primers and their concentrations in each of the PCR multiplexes used for the genetic analysis of *E. camaldulensis* trees.

Multiplex 1	Multiplex 2	Multiplex 3
0.15µM *Eg16* (F/R)	0.15µM *Eg91* (F/R)	0.16µM *Eg96* (F/R)
0.16µM *Eg99* (F/R)	0.17µM *Eg65* (F/R)	0.15µM *Eg84* (F/R)
0.16µM *Eg98* (F/R)		0.17µM *Eg67* (F/R)

2.3.2   Genetic analyses

A number of genetic diversity measures were calculated for the study population. The number of alleles (A) and observed (H_o) and expected (H_e) heterozygosity were estimated using the program GenAlEx (Peakall & Smouse 2005). H_e is a measure of allelic diversity: it represents the level of heterozygosity expected under conditions of random mating, given the allele frequencies observed in the population. Wright's inbreeding coefficient (*f*) measures the probability that both alleles at a locus in an individual are identical by descent. In other words, *f* indicates the deficiency of heterozygotes relative to Hardy-Weinberg expectations. The inbreeding coefficient was calculated in Genepop (Raymond & Rousset 1995) using the method of Weir & Cockerham (1984). While *f* is an important population genetic parameter, it may also help to indirectly identify loci with potential null alleles. The non-amplification of

alleles can lead to an excess of homozygotes being detected in the observed population. By comparing values of f across individual loci, any locus with an extremely large value of f, compared to the rest, can be considered suspect. Linkage disequilibrium amongst loci was also assessed in Genepop using the default Markov-chain parameters provided in the program (Dememorization number = 1000; Number of batches = 100; Number of iterations per batch = 1000).

Several measures allow an assessment of the suitability of a suite of genetic markers for individual identification. Genetic data for the 63 individuals at Tungkillo were assessed for the probability of parentage exclusion (E) and probability of identity (P_{ID}). E is the power of a genetic marker to exclude an individual as the parent of an offspring based on their observed genotypes (Weir 1998) and P_{ID} is the probability that two individuals of a population share the same genotype based on the frequency of alleles observed in a population (Paetkau *et al.* 1995). E was calculated in the program Cervus (Marshall *et al.* 1998) and P_{ID} was calculated in Identity (Wagner & Sefc 1999). The value of E presented is that calculated for when one parent is known and the second parent is to be assigned (as opposed to when neither parent is known) (Weir 1998).

2.3.3   Characterisation of microsatellite allelic diversity in *E. camaldulensis*

The selected eight *E. globulus* microsatellite loci were highly polymorphic across the *E. camaldulensis* study population. Amongst the 63 individuals sampled, a total of 61 alleles were detected at the eight loci, with a mean of 8 ($\pm$ 2.07 s.d.) alleles per locus (Table 2.5). This ranged from five alleles at *Eg16* to 11 alleles detected at *Eg84*. The observed and expected heterozygosity levels for all loci were also high. However, at one locus (*Eg67*) significantly fewer heterozygotes were detected than expected (H_o=0.40 c.f. H_e=0.74), and this was also reflected in the high value of f (inbreeding coefficient). An excess of homozygotes at this locus is potentially indicative of null alleles and, indeed, *Eg67* had proved problematic to amplify during the screening phase suggesting a high frequency of null alleles. Null alleles are undesirable for mating system and parentage analyses, thus, *Eg67* was subsequently dropped from further analyses. Tests for linkage disequilibrium amongst loci were performed on the eight microsatellite loci. Amongst the 28 possible loci combinations, significant linkage was observed between only one combination of loci, *Eg65* and *Eg96* (χ^2 = 10.781, *P*=0.005).

The final panel of seven microsatellite loci (i.e. excluding *Eg67*) allow for very high levels of genetic discrimination between individuals of *E. camaldulensis*. The total multi-locus probability of identity (P_{ID}) was 0.0000026 (Table 2.5), indicating an extremely low probability of encountering two individuals with the same multi-locus genotype. Similarly, the single locus parentage exclusion values were all reasonably high (Table 2.5) and when the seven loci were combined, the total exclusionary power of this set of loci was 0.990. That is, using these genetic markers, an individual can be excluded with 99% certainty from being the parent of a particular offspring, making these markers ideal for parentage testing and population genetic analyses of *E. camaldulensis* trees.

Table 2.5: Genetic diversity of *E. camaldulensis* trees.

N = number of individuals genotyped; A = number of alleles; H_o = Observed heterozygosity; H_e = Expected heterozygosity; f = Wright's inbreeding coefficient; P_{ID} = Probability of Identity; and E = Parentage exclusionary power. Note that the locus *Eg67* was excluded from further analyses due to problems with amplification and is not included in any paternity analyses.

Locus	N	Size range (bp)	A	H_o	H_e	f	P_{ID}	E
Eg99	58	180-198	6	0.62	0.71	0.12	0.193	0.448
Eg98	40	166-181	6	0.85	0.73	-0.16	0.166	0.503
Eg16	57	231-243	5	0.46	0.51	0.10	0.348	0.246
Eg91	53	134-151	8	0.79	0.72	-0.10	0.145	0.491
Eg65	55	231-270	10	0.64	0.64	0.00	0.165	0.428
Eg96	53	274-292	7	0.60	0.56	-0.07	0.203	0.358
Eg84	52	101-131	11	0.88	0.87	-0.01	0.048	0.745
Eg67	25	154-184	8	0.40	0.74	0.46	0.258	0.511
MEAN			**7.63**	**0.66**	**0.68**	**0.04**	**0.191**	**0.466**
TOTAL (ALL LOCI)							**6.64×10^{-7}**	**0.995**
TOTAL (EXCL. *Eg67*)							**2.57×10^{-6}**	**0.990**

2.4 Isolation, development and characterisation of microsatellite markers from *Eucalyptus leucoxylon*

An insufficient number of *E. globulus* microsatellite primers were transferred to *E. leucoxylon* (Section 2.2.2), thus, it was necessary to isolate microsatellites specifically for *E. leucoxylon*. In this section, I describe the isolation of microsatellite-containing sequences from *E. leucoxylon* using a magnetic-bead enrichment method (Gardner *et al.* 1999). PCR primers were developed from DNA sequences flanking the microsatellite region and loci were screened across an initially small number of *E. leucoxylon* individuals to assess their levels of polymorphism. Loci that were polymorphic and amplified reliably were selected for further characterisation in two natural *E. leucoxylon* populations. In addition, the conservation of microsatellite primers was tested in a wide range of eucalypt taxa and two closely related genera.

2.4.1 Development and screening of a microsatellite-enriched genomic library for *E. leucoxylon*

2.4.1.1 *Plant material and DNA isolation*

Total genomic DNA was extracted from leaves of a single *E. leucoxylon* individual (El5602, Flaxley, South Australia) using the protocol of Byrne *et al.* (1993), and subsequently used to develop microsatellite-enriched genomic libraries. Amplification of microsatellite loci and polymorphism levels were initially tested in a small number of *E. leucoxylon* individuals sampled from the MLR and AP region of South Australia (EL01-EL08, Table 2.1). Variation at microsatellite loci was further characterised in 68 individuals from two natural populations of *E. leucoxylon*, located at Flaxley in the Mt Lofty Ranges (301500E/6109400N) (tree and site details provided in Chapter 4) and Ngarkat Conservation Park, located close to the SA-Victoria border, in South Australia. Genomic DNA of these individuals was extracted from leaf material using the MasterPure™ Plant Leaf DNA Purification Kit as per the manufacturer's protocol.

Samples from *Eucalyptus* species in which microsatellites were tested for conservation of loci were collected from trees in the Currency Creek Arboretum (CCA), Waite Arboretum (WA) or the Adelaide Botanic Gardens (ABG), South Australia (Appendix 2). Species were selected on the basis of the classification of Brooker & Kleinig (2001) and their phylogenetic relationships (Steane *et al.* 2002). Leaves were collected from

two individuals of each species and genomic DNA extracted using the MasterPure™ Plant Leaf DNA Purification Kit following the manufacturer's instructions.

2.4.1.2 *Magnetic bead enrichment of genomic libraries from* E. leucoxylon *for microsatellites*

The isolation of microsatellites essentially followed the magnetic bead enrichment method of Gardner *et al.* (1999) described briefly here. Twelve μg of DNA from El5602 was digested with *Sau*3A restriction enzyme and linkers were ligated to the *Sau*3A-cut DNA. Linker-ligated DNA was then size-fractionated on 2.5% Nu Sieve gel (FMC Bioproducts) to create a genomic library of fragments 300-1000bp.

Four oligonucleotide microsatellite probes were trialed on separate occasions using the same magnetic bead enrichment procedure each time, but with modification to the hybridisation temperature dependent on the GC content of the probe. These were $(AAAG)_6$, $(AAC)_6$, $(AC)_{11}$ and $(CAG)_8$. A biotin-labelled microsatellite probe was attached to streptavidin-coated magnetic beads (Magnesphere Paramagnetic Particles, Promega). Linker-ligated DNA was hybridised to the oligonucleotide probe attached to magnetic beads at 55°C (AAAG, AAC, AC probes) or 65°C (CAG probe), with unbound DNA fragments removed from the magnetic bead solution using a series of washes in 1X SSC then 0.5X SSC. Microsatellite-enriched fragments were recovered from the magnetic beads and purified using the Mol Bio PCR purification kit. Long-range PCR was then performed on the recovered fragments using the following protocol: PCR products were amplified in a volume of 50μl (1x Gibco BRL eLONGase Buffer A, 1x Buffer B (final $[MgCl_2] = 1.5mM$), 0.2mM of each dNTP, 0.2μM linker A, 1U Gibco BRL eLONGase polymerase) with the following cycling conditions: 92°C for 1min (1 cycle) and 92°C for 30sec, 60°C for 1min, 72°C for 15 min (30 cycles). For $(AC)_n$ and $(CAG)_n$ microsatellite libraries, the microsatellite-enrichment procedure was repeated on the PCR-amplified fragments to increase the frequency of microsatellite-containing inserts in the genomic library.

Cloning of microsatellite-enriched PCR products was achieved using the plasmid pGEM 5Zf(+) cut with *Eco*R V (pGEM-T Vector System, Promega) following the manufacturer's instructions. Plasmid and insert were transformed in competent JM109

cells (Promega) and recombinant clones were detected using standard blue/white screening methods (Sambrook *et al.* 1989). Positive (insert-containing) clones were re-plated onto duplicate LB plates and incubated overnight at 37°C.

2.4.1.3 Library screening

Two methods were used for screening the microsatellite-enriched library. The first followed the PCR-screening method described in Gardner *et al.* (1999) which was conducted for $(AAAG)_n$ and $(AAC)_n$-enriched libraries. Colonies that contained PCR inserts were lysed in 10mM Tris-HCl (pH 8.0) at 95°C and 1µl of this extract used in a PCR reaction. Inserts were amplified using universal primers (M13 reverse sequencing primer and T7 promoter primer) and a microsatellite primer ($AAAG_6$ or AAC_6) with the following PCR conditions: 1x AmpliTaq Gold PCR Buffer, 4mM $MgCl_2$, 0.2mM each dNTP, 0.2µM each primer and 1U AmpliTaq Gold polymerase in a 50µl reaction volume. PCR amplification was on a Hybaid OMNE 200 thermal cycler with the following cycling conditions: 95°C for 9 minutes (1 cycle), 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds (30 cycles) and final extension at 72°C for 6 minutes (1 cycle). Clones that contained a microsatellite produced two to three bands when the PCR product was visualised by electrophoresis on a 1.5% agarose gel: the larger band represents the product amplified with the universal primers (i.e. the entire insert), the second and third bands represent the products amplified by the microsatellite primer and one or the other of the universal primers.

Due to the low success rate of the $(AAAG)_n$ and $(AAC)_n$ microsatellite-enrichment (Section 2.4.2), I employed a more efficient screening method for the $(AC)_n$ and $(CAG)_n$-enriched libraries. These were screened using standard colony hybridisation techniques (Sambrook *et al.* 1989) and colorimetric detection (Boehringer-Mannheim). This method allowed for a large number of colonies to be screened in a short amount of time. In brief, colonies were baked onto nylon membranes and hybridised to biotin-labelled probes of either $(AC)_{11}$ or $(CAG)_8$ at 55°C (AC) or 65°C (CAG) overnight. Membranes were subjected to a series of washes of increasing stringency (2X SSC, 1X SSC, 0.5X SSC) at temperatures 10°C less than the hybridisation temperature to remove any unbound probe. Membranes were prepared for colorimetric detection and then exposed to Streptavidin/Alkaline Phosphatase conjugate solution (Boehringer-

Mannheim), which binds to the biotin probe. Unbound conjugate was removed from the membranes by washing and then membranes were equilibrated in a detection buffer before being exposed to the colour substrate solution, NBT/BCIP (Boehringer Mannheim). The colour precipitate was left to form for up to 30 minutes. Under stringent screening conditions, only colonies containing the target microsatellite sequence ("positive" colonies) are stained and thus can be identified and selected for further analysis. Positive colonies were lysed in 10mM Tris-HCl (pH 8.0) and PCR amplified for sequencing (Section 2.4.1.4).

2.4.1.4   Sequencing of microsatellite-containing plasmids and primer development

Putative microsatellite-containing inserts were recovered from the plasmids by PCR amplification using the universal M13 and T7 promoter primers under the following conditions: 1x AmpliTaq Gold PCR Buffer, 4mM MgCl$_2$, 0.2mM each dNTP, 0.2μM each primer and 1U AmpliTaq Gold polymerase. PCR amplification was on a Hybaid OMNE 200 thermal cycler with the following cycling conditions: 95°C for 9 minutes (1 cycle), 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds (30 cycles) and final extension at 72°C for 6 minutes. PCR products were purified prior to sequencing using the Mol Bio PCR purification kit. PCR products were sequenced using the Big Dye Terminator Ready Reaction Kit (Applied Biosystems) and analysed on an ABI-Prism 377 automated sequencer. Sequences were edited and aligned by eye in SeAl (version 1.0) to check for duplicate clones.

Primer pairs were designed from the DNA sequence flanking the microsatellite, using the program Oligo (version 4.0), to meet the following conditions: T_m of 58-60°C; GC content 40-60%; no CC/GG ends, no significant hairpins or duplexes, product size 100-400bp.

2.4.1.5   Screening microsatellite primers for polymorphisms

To allow screening of microsatellite primers for polymorphism without directly fluorescently labelling each primer pair, the protocol of Schuelke (2000) was employed. One primer of the microsatellite primer pair is designed with a universal M13 tail attached and a third primer is the reverse complement of M13 with a fluorescent dye label attached at the 5' end (e.g. FAM, TET, HEX). The product was amplified by a

nested PCR protocol in 20μl containing: 1x AmpliTaq Gold PCR buffer, 2-4mM MgCl$_2$, 0.2μM fluorescently-labelled M13 primer, 0.2μM Primer 1 and 0.05μM Primer 2 (with M13 tail), 0.2mM of each dNTP and 0.02U AmpliTaq Gold polymerase. A touchdown PCR program was used with a cycling profile of 95°C for 9 min, 65°C for 45 sec and 72°C for 1min (1 cycle); 94°C for 45sec, 60°C for 45sec and 75°C for 1min (1 cycle); 94°C for 45sec, 58°C for 45sec and 75°C for 1min (1 cycle); 94°C for 45sec, 55°C for 45sec and 75°C for 1min (8 cycles); 94°C for 45sec, 52°C for 45sec and 75°C for 1min (32 cycles) and a final extension step at 72°C for 12 mins. Fluorescently-tagged products were then separated on a 5% denaturing acrylamide gel run on an ABI-Prism 377 automated sequencer as per methods in Section 2.2.1.

Fluorescently-tagged microsatellite markers were initially screened on a panel of seven *E. leucoxylon* individuals sampled from across the study region (EL01-EL08, Table 2.1). The microsatellite markers that showed clearly interpretable polymorphisms and that amplified products of suitable sizes for lane multiplexing were then chosen for direct fluorescent-dye labelling. One primer of each pair was labelled at the 5' end with one of the following dyes: 6-FAM, TET or HEX. Loci for direct labelling were chosen on the basis that their allele size ranges allowed for eight loci to be multiplexed and electrophoresed in a single lane. Microsatellite loci were PCR-multiplexed using a step-down program 94°C for 9 min (1 cycle); 94°C for 30sec, 65-55°C (step down 2°C for each cycle) for 30sec, 72°C for 45sec (10 cycles); 94°C for 30sec, 55°C for 30sec, 72°C for 45sec (20 cycles); final extension 72°C for 12min. PCR multiplex 1 contained *El28*, *El16* and *El23* or *El29*; multiplex 2 contained *El07*, *El14*, *El18*; and multiplex 3 contained *El01*, *El13* (Table 2.6) with amplification under the following conditions: 1x AmpliTaq Gold PCR buffer, 2mM MgCl$_2$, 0.14 - 0.2 μM of each primer, 0.2mM each dNTP and 0.03U AmpliTaq Gold polymerase in a total volume of 15μl.

Table 2.6: *Eucalyptus leucoxylon* microsatellite PCR multiplexes

Details of microsatellite primers and their concentrations in each of the three PCR multiplexes used for the genetic analysis of *E. leucoxylon* trees.

Multiplex 1	Multiplex 2	Multiplex 3
0.15µM *El28* F/R	0.14µM *El07* F/R	0.18µM *El01* F/R
0.16µM *El16* F/R	0.20µM *El14* F/R	0.16µM *El13* F/R
0.16µM *El29* or *El23* F/R	0.16µM *El18* F/R	

2.4.1.6   Genetic data analysis

Microsatellite locus allele frequencies and descriptive locus statistics (A, H_o, H_e, f) in the two natural populations of *E. leucoxylon* surveyed were calculated in GenAlEx (Peakall & Smouse 2005). Linkage disequilibrium was assessed in Genepop (Raymond & Rousset 1995). Genetic information content was estimated by the single-locus and multi-locus probability of genetic identity (P_{ID}) (Paetkau *et al.* 1995) and the paternity exclusion probability (E) (Weir 1996), as described in Section 2.3.2.

2.4.1.7   Cross species amplification of microsatellite loci

Cross-species amplification of microsatellite primers was attempted in a range of *Eucalyptus* species of increasing evolutionary distance from *E. leucoxylon*. Species from five of the most speciose sections of *Symphyomyrtus*, from four of the five subgenera of *Eucalyptus* and from the two *Eucalyptus*-like genera, *Angophora* and *Corymbia*, were chosen for testing (note that the status of *Angophora* and *Corymbia* is still unclear but I have followed the relationships of *Eucalyptus* demonstrated in Steane *et al.* 2002). The locations of voucher specimens of each species are provided in Appendix 1. Two individuals from each species were tested using the step-down PCR program described previously (section 2.4.1.5) but with an incremental step down from 65°C to 50°C. Microsatellite loci were presumed to be present in non-target species if the primers amplified a product of approximately the same size as found in *E. leucoxylon* that was visible when run on a 1.5% agarose gel. I also examined the relationship between evolutionary distance and the degree of microsatellite conservation among the taxa compared. Sequences of the nuclear rDNA cistron comprising the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA gene were used to construct a genetic distance matrix to determine the evolutionary distances amongst eucalypt species. The majority of sequences were obtained from Steane *et al.* (2002),

available on GenBank (http://www.ncbi.nlm.nih.gov). Species for which rDNA
sequence data were not already available (*E. leucoxylon* and *E. petiolaris*) were
sequenced using the ITS4 and ITS5 primers of White *et al.* (1990), using sequencing
conditions described above (section 2.4.1.4). A Neighbour-Joining tree constructed
from pair-wise Kimura 2-parameter distances was used to visually display evolutionary
distances among the taxa.

2.4.2   Results of microsatellite isolation procedure

2.4.2.1   (AAAG)$_n$ tetranucleotide repeats

113 colonies were screened using the PCR-based screening method. Of these, 19
colonies (17%) produced multiple bands and were selected for sequencing. Upon
inspection of the resulting DNA sequences it was clear that a number of fragments were
AG-rich but contained no microsatellite region, while in several other cases duplicates
were found. A number of microsatellite-like regions were found, however, they were
complex and interrupted repeats that would be unlikely to follow a consistent 4bp
stepwise pattern in allele lengths. No AAAG microsatellites were selected for further
development.

2.4.2.2   (AAC)$_n$ trinucleotide repeats

263 colonies enriched for AAC microsatellites were screened using the PCR-based
method. Of these, 56 colonies (21%) showed multiple bands and were chosen for
sequencing. Again, a number of duplicate sequences were detected and a range of
complex, imperfect microsatellites were found also. Four AAC perfect repeats were
detected, however, in all cases these microsatellites contained only 4-6 repeats, hence
these loci were unlikely to be polymorphic. Two imperfect microsatellites of sufficient
length were found, but were located too close to the *Sau*3A cut site so there was
insufficient flanking sequence available in which to develop primers. No AAC
microsatellites were selected for further development. Again, a second round of
enrichment with a higher hybridisation temperature may have increased the yield of
AAC microsatellites.

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2.4.2.3   *(CAG)$_n$ trinucleotide repeats*

Due to the low efficiency of the microsatellite-enrichment procedure for (AAAG)$_n$ and (AAC)$_n$ microsatellites I performed two rounds of magnetic bead enrichment for (CAG)$_n$ microsatellites. 519 microsatellite clones were screened using the colony hybridisation method described above. 52 (10%) strong positives were identified, of which, 23 were sequenced. Of these, four clones were false positives and no duplicate sequences were found. Four of the sequences recovered had insufficient DNA sequence flanking the microsatellite region, therefore, primers could not be developed for these. Almost half of the microsatellites sequenced were compound repeats (i.e. made up of two or more repeat types).

2.4.2.4   *(AC)$_n$ dinucleotide repeats*

Again, two rounds of enrichment were performed for (AC)$_n$ microsatellites and clones screened using the colony hybridisation method. Of the 592 microsatellite clones from the (CA)$_n$ enrichment that were screened, 58 (10%) strong positives were identified. Of these, 46 clones were sequenced. Six clones were CA-enriched but contained no microsatellite and five clones were duplicates of two other clones; the rest of the microsatellite sequences were unique. Almost half of the microsatellites recovered were compound repeats.

2.4.2.5   *Overview of the microsatellite isolation procedure*

For all motifs, a low rate of microsatellite-enrichment of genomic libraries was found. This was essentially 0% for (AAAG)$_n$ and (AAC)$_n$ microsatellites (as no perfect repeats were found) and approximately 10% after two rounds of enrichment for (AC)$_n$ and (CAG)$_n$ microsatellites. These figures are much lower than that reported in a number of other studies that used microsatellite-enrichment procedures (reviewed in Squirrell *et al.* 2003). A number of adjustments can be suggested that may have increased the efficiency of microsatellite enrichment.

No useable microsatellites were detected in the (AAAG)$_n$ and (AAC)$_n$ enriched genomic libraries. Several authors have indicated that tetranucleotide repeats are rare in plants (Toth *et al.* 2000, Lagercrantz *et al.* 1993, Edwards *et al.* 1996), which may be the case for *E. leucoxylon*. However, a number of improvements to the isolation procedure for

these motifs may be suggested. In both cases, AG- and AC-rich DNA sequences were recovered during the screening process. This tends to indicate that the temperature for hybridisation of *Sau3a*-cut DNA to the microsatellite probe on the magnetic beads was too low. A higher hybridisation temperature would have increased the specificity of DNA binding, increasing the yield of these microsatellites. In addition, a second round of microsatellite enrichment appeared to increase the yield of $(AC)_n$ and $(CAG)_n$ microsatellites and would be useful for other probes. Lastly, it also appears that the washing procedure used to wash non-specific sequences from the magnetic beads may have been insufficient. Increasing the number of or the time of high stringency washes may also have helped to increase the proportion of microsatellite-containing sequences being recovered.

2.4.3   Microsatellite primer development and initial screening in *E. leucoxylon*

Primer pairs were designed from the flanking sequence of 20 $(AC)_n$ microsatellites and 9 $(CAG)_n$ microsatellites. All unique microsatellite sequences were deposited with GenBank (http://www.ncbi.nlm.nih.gov).

The 29 microsatellite loci were initially screened in a small sample of *E. leucoxylon* individuals to determine primarily whether primer pairs amplified products consistent with the cloned microsatellite and whether the microsatellite loci were polymorphic. At the end of this process, eleven primer pairs were discounted because they showed non-specific amplification of secondary bands, they were monomorphic or potentially contained null alleles (Table 2.7).

Of the remaining 18 primer pairs, eight (*El01, El07, El13, El14, El16, El18, El23* and *El28*), were selected for further use, based on their level of polymorphism, repeatability, their lack of stutter and appropriate size range for lane multiplexing (Table 2.8). These loci were fluorescently-labelled in such a way that allowed for all eight loci to be electrophoresed in a single lane and to be PCR-multiplexed (detailed in section 2.4.1.5).

Table 2.7: Details of microsatellite loci isolated from *E. leucoxylon* and results of initial testing.

Locus Name	Motif	Screening result	Genbank Accession No.
El01	$(GT)_{10}$	Good, polymorphic	AY390559
El02	$(AC)_{24}$	Null alleles?	AY390560
El03	$(AC)_{16} (AT)_5 (GTAT)_9$	Null alleles?	AY390561
El04	$(AC)_5 T (GC)_4 (AC)_{20}$	Null alleles?	AY390562
El05	$(TA)_7 (GA)_1 (CA)_7 (TA)_2$	Inconsistent sizing	AY390563
El06	$(AC)_{12}$	Good, polymorphic	AY390564
El07	$(GT)_{13}$	Good, polymorphic	AY390565
El08	$(AT)_6 (GT)_{10} (TT)_2 (GT)_5$	Good, low polymorphism	AY390566
El09	$(TG)_{12} (CG)_1 (GA)_{12}$	Failed to amplify	AY390567
El10	$(AC)_9$	Inconsistent sizing	AY390568
El11	$(AT)_2 (GT)_{26} (AT)_4$	Inconsistent sizing	AY390569
El12	$(AT)_3 (GT)_{13}$	Null alleles?	AY390570
El13	$(TC)_{17} (AC)_{10}$	Good, polymorphic	AY390571
El14	$(GT)_{13} (AG)_{15}$	Good, polymorphic	AY390572
El15	$(AC)_{11}$	Bad PCR product	AY390573
El16	$(AT)_6 (GT)_{20}$	Good, polymorphic	AY390574
El17	$(GT)_{18} (GA)_9$	Null alleles?	AY390575
El18	$(TC)_{10} (AC)_8 (TT)_1 (CA)_2$	Good, polymorphic	AY390576
El19	$(AC)_8$	Monomorphic	AY390577
El20	$(AC)_9$	Monomorphic	AY390578
El22	$(GCC)_4 (CAG)_{13}$	Good, polymorphic	AY390579
El23	$(GCT)_7$	Good, polymorphic	AY390580
El24	$(CTG)_5 (CTT)_3$	Monomorphic	AY390581
El25	$(CAG)_9$	Bad PCR product	AY390582
El26	$(CTG)_6 (CTT)_4$	Monomorphic	AY390583
El27	$(CTG)_{12}$	Good, low polymorphism	AY390584
El28	$(CAG)_8$	Good, polymorphic	AY390585
El29	$(AGC)_7$	Good, polymorphic	AY390586
El30	$(AAC)_3 (AGC)_7$	Monomorphic	AY390587

However, after testing the eight-locus multiplex across two populations of *E. leucoxylon* individuals (section 2.4.4), it was found that *El23* produced spurious banding patterns that were likely due to co-amplification of a second locus. *El23* was subsequently replaced by *El29* in the lane-multiplex without necessitating the rearrangement of the remaining seven loci.

A summary of the attrition rates of $(CAG)_n$ and $(AC)_n$ microsatellite loci from isolation, through primer design and testing, to useable loci is presented in Table 2.9. A review paper by Squirrell *et al.* (2003) found an average 83% attrition rate from successfully sequenced clones to useable polymorphic loci. The values presented here from *E. leucoxylon* are very similar (81% for $(CAG)_n$ microsatellites and 85% for $(AC)_n$ microsatellites).

Table 2.8: Primer details of the final set of *E. leucoxylon* microsatellite loci.

All loci amplified cleanly and were polymorphic amongst two populations of *E. leucoxylon*. The final set of loci was suitable for PCR- and lane-multiplexing.

Locus	Repeat motif	Primer sequence (5' - 3')	Product length (bp)[a]
El01	$(GT)_{10}$	(F) FAM -CACCTAGTTGCTTTCAGAC (R) CCTGATAAAAGCAATAAAGCAG	362
El07	$(GT)_{13}$	(F) TET -TGGAGATAGTCACGGCAAC (R) CCCAGTTGGTATTCCTTAG	121
El13	$(TC)_{17}(AC)_{10}$	(F) HEX -CAAGAGTCACAGCCAAGCC (R) GACAACGCATCTTTCTTTCTG	190
El14	$(GT)_{13}(AG)_{15}$	(F) ACCTTAGAAAAGTCGAAGCATC (R) FAM -ACCTCCACATACCAGTCAC	184
El16	$(AT)_6(GT)_{20}$	(F) HEX -GATTTATACCTCATTTGTCGC (R) ACCCTACAGCAGAAGCATAC	246
El18	$(TC)_{10}(AC)_8(CA)_2$	(F) ACCCACCACCTCTGTTCAC (R) TET-CAGAGTCCATGAACGCAAG	288
El23	$(GCT)_7$	(F) FAM-AATACACGAAATGCCACAAAC (R) CTCCAAAAACCAGTTCTCAG	281
El28	$(CAG)_8$	(F) TCAGTAGGAGGGGCTAGAC (R) TET-GTAGGAGAGTCCAGTTCGC	215
El29	$(GA)_{14}$	(F) FAM-CTTTCATGTCCTTCACCAATC (R) ATTAGGGTTTTGAAGCGTCTC	268

[a] Size of PCR product in original target individual EL5602.

Table 2.9: Rate of attrition of suitable *E. leucoxylon* microsatellites from screening through to use (after Squirrell *et al.* 2003), emphasising the rate of attrition from the number of clones successfully sequenced to the number of useable loci.

Motif	Clones screened	Positive [a]	Sequenced [b]	Contain m'sat [c]	Duplicates [d]	Suit primer design [e]	Primers designed [f]	Primers amplified [f]	Mono-morphic [g]	Useable loci [h]
$(AC)_n$	592	58 (9.8%)	**46** (79%)	40 (87%)	5 (11%)	20 (43%)	20	19	2	7 (15%)
$(CAG)_n$	519	52 (10%)	**23** (44%)	21 (91%)	0 (0%)	15 (65%)	9	9	3	4 (19%)

[a] Of the number of clones screened, the number of clones that were identified as potentially containing microsatellites ("positives") during the screening process;

[b] Of the number of positive clones, the number for which sequence data was obtained (the rate of attrition includes failure of clones to PCR amplify or sequence and a proportion that were not PCR'd or sequenced because sufficient microsatellites were already available);

[c] Of the number of clones sequenced, the number of clones that contained a microsatellite (rate of attrition includes sequences that contained an interrupted or otherwise unusable microsatellite sequence);

[d] Of the number of clones sequenced, the number of clones that were duplicates (i.e. the same microsatellite was recovered more than once);

[e] Of the number of clones sequenced, the number of clones that contained sufficient sequence data flanking the microsatellite in which to design primers (rate of attrition includes clones where insufficient flanking region was available or the flanking sequence was unsuitable for primer design (e.g. repetitive sequence);

[f] The number of primer pairs designed for unique microsatellites and the number of primers that successfully amplified in all samples in which they were initially tested (n=7)

[g] The number of loci that were invariant in all samples in which they were initially tested

[h] Of the number of clones sequenced, the number of clones that contained microsatellites that were highly polymorphic and which produced banding patterns that were easily interpretable (the rate of attrition includes loci that were difficult to interpret due to stutter bands or spurious banding due to co-amplification of a second locus, or low levels of polymorphism).

2.4.4 Characterisation of microsatellite allelic diversity in *E. leucoxylon*

Across the nine microsatellite loci screened in 68 individuals from the two study populations of *E. leucoxylon,* a total of 111 alleles were identified, with an average of 12.3 alleles per locus (range 8-20) (Table 2.10). Five of the nine microsatellite loci described in Table 2.10 are perfect repeats, the remaining four are compound repeats. Compound repeats were significantly more polymorphic (average 15.5 alleles/locus) than the perfect repeats (9.8 alleles/locus) (t=3.01, df=7, P=0.02).

For the final eight-locus multiplex (i.e. excluding *El23* and including *El29*), the mean expected heterozygosity was 0.83 and the mean observed heterozygosity was 0.72 (Table 2.10). The paternity exclusion probability (E) was high for most loci, with the exception of *El29*, perhaps due to a small sample size. The combined probability of paternity exclusion using all eight loci was 0.9990, indicating a 99.9% chance of correctly excluding a random non-parent individual tree in the population. The single-locus probability of genetic identity (P_{ID}) varied from 0.023 to 0.413 (*El29*, due to small sample size) with a combined value of 6.37 x 10^{-10}, allowing an extremely high level of individual identification. The eight-locus multiplex provides a very high level of discrimination, which makes these markers ideal for parentage-type studies in *E. leucoxylon.*

All loci besides *El18* and *El28* exhibit a positive value of *f,* suggesting inbreeding in the two *E. leucoxylon* populations. *El23* and *El29* have very positive *f* values, which may indicate the presence of null alleles. However, as mentioned previously, *El23* was discontinued due to spurious banding patterns and the positive *f* for *El29* is potentially due to small sample size. In mating system studies of *E. leucoxylon,* null alleles were not detected for any of the loci across a number of open-pollinated families (Chapter 6). Linkage disequilibrium tests revealed significant associations between four possible combinations of the eight loci out of a possible 42 combinations in the sample of 68 *E. leucoxylon* individuals (Table 2.11). However, for the genetic analyses conducted in the forthcoming chapters no significant linkage disequilibrium was detected in the sample of 28 adult paddock trees surveyed at Flaxley (data not shown).

Table 2.10: Genetic diversity measures of *E. leucoxylon* trees

Locus name, allele size range (bp), number of alleles detected per locus (number of individuals genotyped), expected heterozygosity (H_e), observed heterozygosity (H_o), inbreeding coefficient (*f*), probability of paternity exclusion (*E*) and probability of genetic identity (P_{ID}).

Locus	N	Allele size range (bp)	A	H_e	H_o	*f*	E	P_{ID}
El01	64	350-406	9	0.74	0.64	+0.132	0.570	0.140
El07	37	107-165	14	0.89	0.70	+0.213	0.582	0.111
El13	65	172-204	13	0.81	0.71	+0.122	0.649	0.084
El14	64	166-208	16	0.92	0.86	+0.067	0.839	0.023
El16	63	222-248	13	0.86	0.68	+0.207	0.742	0.055
El18	68	279-319	20	0.90	0.93	-0.024	0.804	0.032
El23	43	270-317	9	0.85	0.47	+0.452	0.621	0.106
El28	65	191-236	9	0.70	0.71	-0.011	0.518	0.176
El29	16	158-282	8	0.77	0.56	+0.276	0.235	0.413
Mean			**12.3**	**0.83**	**0.70**	**+0.16**	**0.62**	**0.13**
Total (All loci)							**0.99996**	**6.77×10^{-11}**
Total (Excl. *El23*)							**0.99990**	**6.37×10^{-10}**

Table 2.11: *El* microsatellite locus combinations exhibiting significant linkage disequilibrium

Locus combination	P
El14 & El18	0.02
El01 & El18	0.04
El13 & El14	0.00
El28 & El16	0.02

2.4.5   Cross-species amplification of microsatellite loci

Eucalyptus leucoxylon microsatellite primer pairs were screened in a range of *Eucalyptus* species and species from two closely related genera, *Corymbia* and *Angophora* (Table 2.12), for the conservation of microsatellite primer sites. As Figure 2.1 shows, the conservation of microsatellite primers tended to decline with increasing evolutionary distance from the target species *E. leucoxylon*. *Eucalyptus leucoxylon* is placed within the section Adnataria, within the subgenus *Symphyomyrtus* (Figure 2.1). All *E. leucoxylon* primer pairs amplified a product of expected size (100%) in species within the section Adnataria. Across sections within the subgenus *Symphyomyrtus* an average of 76% of primers were conserved. Across the *Eucalyptus* subgenera tested, 53% of microsatellite primers were conserved but only 22% of primers were conserved in the more distantly related genera *Angophora* and *Corymbia*. Details of the loci that amplified in each species are provided in Table 2.12.

2.5   Discussion

The final seven-locus microsatellite multiplex developed for *E. camaldulensis* and the final eight-locus microsatellite multiplex developed for *E. leucoxylon*, provide for very high levels of individual identification in natural populations of each species. They provide sufficient genetic information for the estimation of a range of population genetic parameters as well as for the estimation of mating system parameters, including determination of such variables as outcrossing rate, average pollination distances and the average number of mating partners. In addition, these loci are PCR-multiplexed and able to be electrophoresed in a single lane, allowing for cost-effective, rapid throughput genetic analysis. Finally, the high level of transferability of *E. leucoxylon* microsatellite primers within the eucalypt subgenus *Symphyomyrtus* suggests that these primers may be relatively easily used in studies involving a wide range of *Eucalyptus* species. Importantly, these are the first microsatellites developed for a group of Eucalypts (section Adnataria) that contains many woodland species that are under threat from habitat fragmentation and ongoing deforestation in south-eastern Australia (e.g. *E. albens*, Prober & Brown 1994; *E. melliodora*, Burrows 2000; *E. largiflorens*, George *et al.* 2005; *E. tricarpa*, Mac Nally & Horrocks 2002).

Table 2.12: Conservation of *E. leucoxylon* microsatellite primers in a range of *Eucalyptus* species and two closely related taxa (*Angophora* and *Corymbia*).

Primers were tested in two individuals of each species: "+" indicates primers amplified a product of similar size to that in *E. leucoxylon* and "–" indicates the primers failed to amplify a product. "+/-" indicates primers amplified in one individual and not the other.
Further characterisation would be required to confirm the presence of homologous microsatellite loci in the non-target species.

Species	Subgenus	Section	El01	El07	El13	El14	El16	El18	El23	El28	No. conserved
E. leucoxylon	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	+	8
E. leucoxylon ssp. pruinosa	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	+	8
E. petiolaris	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	+	8
E. melliodora	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	+	8
E. microcarpa	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	-	7
E. camaldulensis	Symphyomyrtus	Exsertaria	+	+	+	+	+	+	+	+	8
E. fasiculosa	Symphyomyrtus	Adnataria	+	+	+	+	+	+	+	+	8
E. salmonophloia	Symphyomyrtus	Bisectaria	+	-	+	+	+	+	-	-	5
E. grandis	Symphyomyrtus	Latoangulata	+	-	+	+	+	+	+	+	7
E. viminalis	Symphyomyrtus	Maidenaria	+	+	+	+	+	+	+	-	7
E. globulus	Symphyomyrtus	Maidenaria	-	+	+	+	+	+	+	-	6
E. nitens	Symphyomyrtus	Maidenaria	+	-	+	+	+	+	+	+	7
E. sieberi	Eucalyptus	Cineraceae	-	-	+	-	+	+	+	-	4
E. obliqua	Eucalyptus	Eucalyptus	+	+/-	+	-	+	-	+	-	4.5
E. marginata	Eucalyptus	Monocalyptus	-	+	+	-	+	-	+	-	4
E. cloeziana	Idiogenes		+	-	+	-	+	-	+	-	4
E. royceii	Eudesmia		+	-	+	+	+	+/-	-	-	4.5
E. eudesmoides	Eudesmia		-	+	-	-	+	+/-	-	-	2.5
E. baileyana	Eudesmia		-	-	+	-	+	-	-	-	2
Angophora costata			+	-	+	-	-	-	-	-	2
Corymbia calophylla			+/-	-	+	-	-	-	-	-	1.5
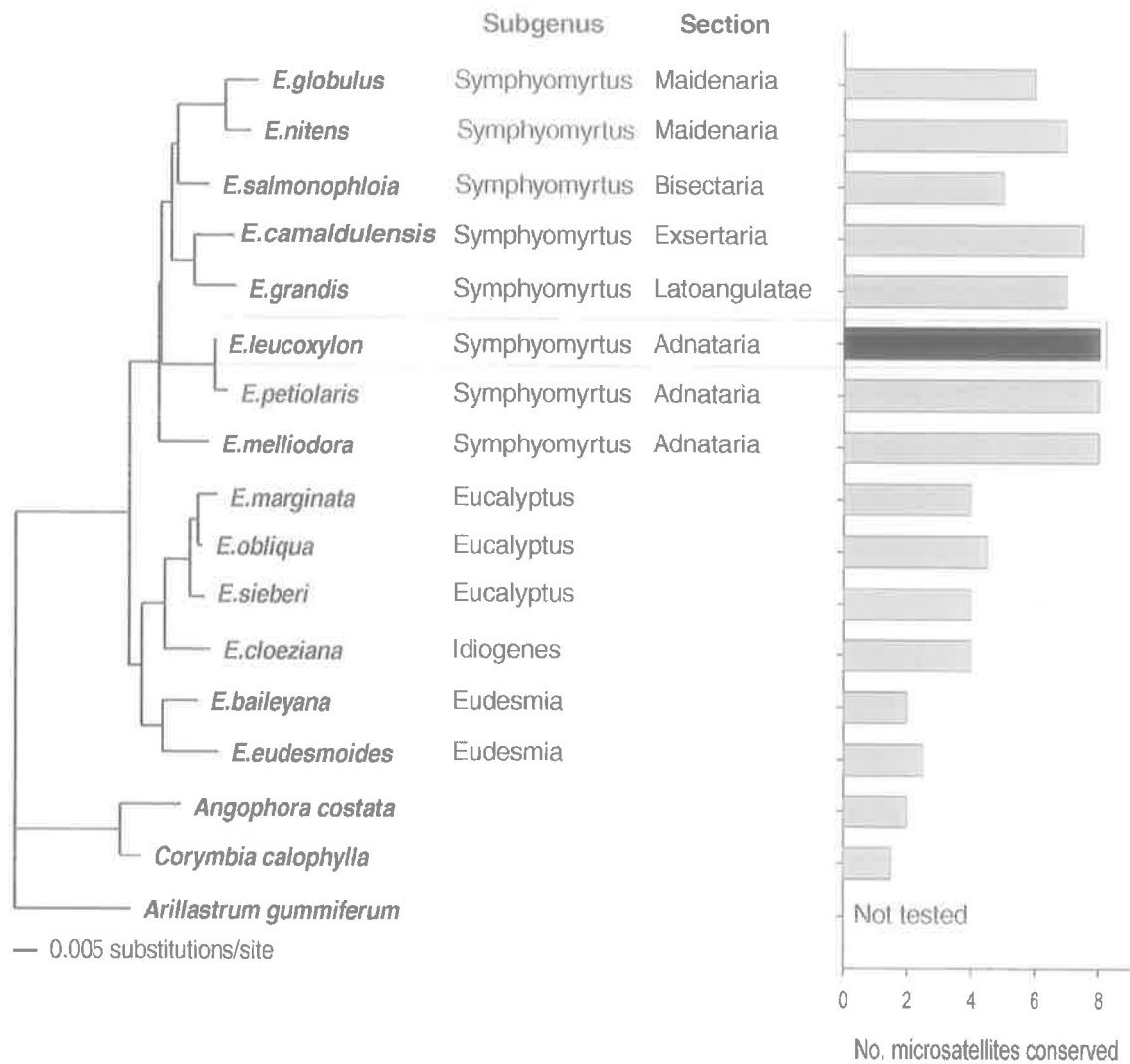
Figure 2.1: **Taxonomic relationships of *Eucalyptus* taxa in which *E. leucoxylon* microsatellites were tested and the number of microsatellites conserved in each species.**

The neighbour-joining tree was constructed using ITS and 5.8S rRNA sequences available from GenBank - *Arillastrum gummiferum* (outgroup) AF058454; *C. calophylla* AF390460; *A. costata* AF058455; *E. eudesmoides* AF390468; *E. baileyana* AF390467; *E. cloeziana* AF058462; *E. sieberi* AF058495; *E. obliqua* AF058484; *E. marginata* AF390530; *E. melliodora* AF390514; *E. petiolaris* AY388998; *E. leucoxylon* AF388997; *E. grandis* AF058475; *E. camaldulensis* AF058473; *E. salmonophloia* AF390509; *E. nitens* AF058472; *E. globulus* AF058463.

Chapter 3   The potential for somaclonal variation in microsatellite profiles in large, long-lived plants

3.1 Introduction

In plants, the meristem is a mitotically derived lineage of undifferentiated cells that produces primary growth and gives rise to all differentiated plant structures. Thus, gametes may arise from cell lineages that have undergone many mitotic divisions. If errors of DNA replication accompany mitosis and are not selectively removed during vegetative growth of the plant (e.g. by cell lineage sorting), they can potentially be passed on to the gametes (Klekowski 1988). This may be especially so for large, long-lived plants (Whitham & Slobodchikoff 1981; Klekowski & Godfrey 1989) and is a potential source of error when estimating outcrossing rates or performing parentage assignments in long-lived plants using highly variable markers such as microsatellites.

Somatic mutations that affect morphological traits are easily observed, and are, therefore, relatively well known in plants. For example, chlorophyll deficiency has been examined in several species (Klekowski & Godfrey 1989; Korn 2001) and many cultivated varieties of plants are the result of somatic mutations (Hartman & Kester 1975; Skirvin *et al.* 1994). Over the last few decades, advances in DNA technology and tissue culture techniques have allowed researchers to more fully document the types and occurrence of somatic mutations at the genetic level. While some somatic mutations may be pre-existing in a plant, mutations can also occur due to biological stresses encountered by plant tissues during the culturing process (Cassells & Curry 2001; Endemann *et al.* 2001) or may be induced by deliberate exposure to mutagens such as X-rays, radiation or UV (Kovalchuk *et al.* 2000; Ahloowalia & Maluszynski 2001). Some examples of the types of somatic mutations occurring in the genome detected during tissue culture include cytological abnormalities (e.g. polyploidy, aneuploidy) (Tremblay *et al.* 1999; Endemann *et al.* 2001), sequence changes (de Laia *et al.* 2000; Kovalchuk *et al.* 2000; Pluhar *et al.* 2001; Kovalchuk *et al.* 2003), and gene activation or silencing (e.g. Larkin *et al.* 1984; Chatterjee & Gupta 1997).

In plant populations, somatic mutations can be a significant source of new genetic variation both within and between individuals (Slatkin 1985; Schneller *et al.* 1998).

This has been shown to be especially so for plants which reproduce clonally (Tuskan *et al.* 1996; Wolf *et al.* 2000; Crespan 2004). The genetic mosaicism hypothesis developed by Whitham & Slobodchikoff (1981) and Gill *et al.* (1995) explores the evolutionary significance of somatic mutations for individuals, especially in the context of how long-lived trees may evolve resistance to short-lived pests. The authors suggest that an advantageous mutation has the potential to become fixed in an individual when there is positive selection for the mutation (for example, plant tissues containing novel chemical defences avoid predation and may therefore become dominant within the canopy). The mutation may then be heritable if gametes are produced from the mutant-containing cell lineage. Not all mutations will be beneficial and mutant cell-lineages may also be purged through natural selection or genetic drift or they may exist in a state somewhere between extinction and fixation (Fagerstrom *et al.* 1998; Pineda-Krch & Fagerstrom 1999; Pineda-Krch & Lehtila 2002).

We should expect to be more able to detect somatic mutations in large, long-lived plant species, as opposed to annual species, simply because of the large number of mitotic divisions that have occurred in the plant over time and the potential for mutations to accumulate over the life span of the plant. Theoretical treatments suggest that the opportunities for mutant cell lineages to spread within an organism are relatively rare, but that the large number of cell divisions in large and long-lived organisms work in favour of creating a genetically heterogenous soma (Antolin & Strobeck 1985; Otto & Hastings 1998; Pineda-Krch & Lehtila 2002). Indeed, there is some evidence that mutation rates for several different types of genetic mutations are higher in longer-lived than short-lived species (e.g. self-sterility, Klekowski 1988; albinism, Klekowski & Godfrey 1989; microsatellites, Udupa & Baum 2001).

3.1.1 Mitotic mutations at microsatellite loci

Microsatellite DNA regions have mutation rates that are, on average, several orders of magnitude higher than that of other DNA markers (Wolfe *et al.* 1987; Hancock 1999). Length variation in microsatellite regions evolves through slippage during the DNA replication process and most commonly results in the addition of one repeat unit (Udupa & Baum 2001). Meiotic microsatellite mutation rates have been estimated to be between 10^{-2} and 10^{-5} per generation for plants (Udupa & Baum 2001; Vigouroux *et al.*

2002). As yet it is unknown whether mitotic mutation rates should be the same or more or less than meiotic rates, though we may expect DNA mismatch repair to function equally as well during mitosis as meiosis. Strand *et al.* (1993) found that the rates of microsatellite mutation were similar in mitotic and meiotic yeast cells, despite the much higher rate of recombination that occurs during meiosis. O'Connell & Ritland (2004) found a single somatic mutation amongst 80 Western Red cedar trees sampled at eight microsatellite loci and estimated the somatic mutation rate in these trees to be 6.3×10^{-4} mutations per locus per generation. While Cloutier *et al.* (2003) found no mutations at twelve microsatellite loci surveyed across twelve *Pinus strobus* genets, based on anatomical and growth data the somatic mutation rate was estimated to be between 9.9×10^{-7} and 3.3×10^{-6} mutations per cell division per locus. These estimates suggest that mitotic mutation rates may be within the range detected for meiotic mutations.

3.1.2 Implications for mating system studies

It is generally assumed that individuals are genetically homogeneous, though this is rarely tested (Pineda-Krch & Lehtila 2004) and the consequences for individual genetic analyses rarely considered. Somatic variation in microsatellite profiles may be problematic for population genetic studies of long-lived species, especially for mating system and parentage-type analyses. A common practice in mating system and parentage studies is to sample leaf (or other tissue) from a tree to determine the maternal genotype. The genotypes of seeds collected from different parts of the tree are compared to the maternal genotype to determine if the seed is the product of self- or cross- fertilisation. If there is variation in microsatellite profiles within a tree, a seed collected from a branch containing a mutation may then produce a misleading genetic profile when compared with the maternal genotype. If the mutant allele is considered a non-maternal allele, the seed will be falsely scored as being outcrossed. In addition, paternity may be falsely assigned or not assigned at all if no matching genotype is found in the population. Alternatively, if it appears that the seed contains no maternal alleles (i.e. a mutant allele plus an outcrossed allele) the seed genotype may be discarded as being an error (e.g. mis-labelling or mis-scoring error).

3.1.3 Aims

Before beginning a full-scale investigation into the mating system of *Eucalyptus camaldulensis* and *E. leucoxylon* (both large and long-lived woodland tree species), I investigated the potential for somatic mutations at microsatellite loci. Somatic tissue was collected from various positions in trees of each of the two species and genotyped at eight microsatellite loci for each species. Within-tree microsatellite genotypes were compared to confirm the consistency of microsatellite profiles across the canopy of individual trees.

3.2 Methods

3.2.1.1 Tissue collection

Leaf material was collected from four individuals of each species from study populations located at Tungkillo (*E. camaldulensis)* and Flaxley (*E. leucoxylon)* in the MLR, South Australia. Both species are large woodland trees that, through habitat clearance, are now predominantly found as scattered individual trees in agricultural regions. In this type of environment, both trees commonly reach heights of up to 25m and the canopy can similarly span 25m in width (Table 3.1). For each individual, leaf material was collected from up to 10 different major branches around the canopy at different heights. Leaves were also collected from the internal as well as external canopy.

Table 3.1: Size measurements of *E. leucoxylon* and *E. camaldulensis* trees sampled for somatic mutations.

All size measurements were made in the field. The circumference of trees (C) was measured at ~1.3m height and DBH calculated from the equation DBH = C/π. Height was estimated visually to the nearest metre (as described in Chapter 4). Canopy diameter was measured at the widest point (*1*) and at 90° to the widest point (*2*).

Tree ID	*DBH* (cm)	Height (m)	Canopy diameter*1* (m)	Canopy diameter*2* (m)
E. leucoxylon				
El001	18	11	11	5
El002	60	13	20	16
El003	73	14	20	21
El005	80	12	12	13
E. camaldulensis				
Ec3278	136	16	22	24
Ec3328	136	16	20	16
Ec3311	166	21	30	29
EcA	127	18	22	23

3.2.1.2   DNA protocols

Genomic DNA was extracted from approximately 300mg of leaf tissue using the MasterPure™ Plant Leaf DNA Purification Kit as per the manufacturer's protocol. Details of the eight primers used to amplify microsatellite loci in *E. camaldulensis* are provided in Chapter 2, Section 2.3. Details of the eight primers used to amplify microsatellite loci in *E. leucoxylon* are provided in Chapter 2, Section 2.4.

Microsatellite loci for *E. camaldulensis* were PCR amplified in a volume of 10μl in three different multiplexes (Table 2.4) and *E. leucoxylon* microsatellites were amplified in a volume of 15μl in three multiplexes also (Table 2.6). All multiplexes contained 1x Applied Biosystems Ampli-Taq Gold PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl), 1.5-2mM $MgCl_2$, 2.5mM each dNTP, 0.16-0.2μM each primer (F/R), 0.02-0.03U Applied Biosystems Ampli-Taq Gold and sterile dH_2O. *Eucalyptus camaldulensis* microsatellite loci were amplified on an Hybaid OMNE-200 thermal cycler using the following cycling conditions: 94°C for 9min, 55°C for 45sec, 72°C for 1min (1 cycle); 94°C for 45sec, 55°C for 45sec, 72°C for 1min (34 cycles) and a final extension step of 72°C for 6min. *Eucalyptus leucoxylon* microsatellite loci were amplified on an Eppendorf Mastercycler thermal cycler using the following cycling conditions: 94°C for 9 min (1 cycle); 94°C for 30sec, 65-55°C (step down 2°C for each cycle) for 30sec, 72°C for 45sec (10 cycles); 94°C for 30sec, 55°C for 30sec, 72°C for 45sec (20 cycles); final extension 72°C for 12min.

Fluorescently-labelled PCR products were analysed on a 5% denaturing acrylamide gel (Long Ranger, 6M Urea/1xTBE) run on an ABI-Prism 377 automated sequencer using the GeneScan application. The output was analysed using Genotyper software (ABI). Products were sized using the TAMRA500 (ABI) size standard.

A number of *E. camaldulensis* samples were run across several acrylamide gels with appropriate positive controls to allow for differences in electrophoretic mobility between gels. All *E. leucoxylon* samples were run on a single gel. In all cases, the electrophoretic profile of the TAMRA size standard was checked closely to account for minor differences in size-calling of the PCR products. In a few cases, small (e.g. 1bp) differences in product sizes between samples were due to differences in migration of the

smaller fragments of the size standard. Any ambiguous products were re-amplified and run again.

3.3 Results

3.3.1 Microsatellite profiles of *E. camaldulensis* and *E. leucoxylon* trees

There was very little variation detected in microsatellite profiles within the four *E. camaldulensis* trees (Table 3.2). In the majority of samples, all within-tree replicates produced identical microsatellite profiles across all loci. The exception was for tree Ec3328 at locus *Eg67* – three out of the nine replicates failed to amplify one of the alleles present in the other six replicates. The three anomalous samples were amplified twice, both times with the same resultant profile. In addition, replicate 1 of Ec3328 failed to amplify a product at the *Eg91* locus despite two separate attempts. These results potentially indicate a mutation in the primer binding region for these loci that prevent the amplification of the microsatellite loci (i.e. null alleles).

There was little variation in microsatellite profiles of *E. leucoxylon* trees also (Table 3.3). Two potential 1bp shifts in microsatellite profiles were detected. The first was at locus *El07* for El002-9 (Figure 3.1). Samples El002-1 to El002-8 produced identically shaped peaks but El002-9 produced a peak that had additional stutter bands. Without prior knowledge or experience of the locus, I would have scored the alleles as 122/128 (the highest peaks) instead of 121/127. Similarly, sample El003-1 at locus *El13* (Figure 3.2) produced a product that was 1bp smaller than the remaining samples, although in practice this peak would have been rounded up and scored as 200/200. As Figure 3.2 shows, the peak height of this sample (values on the Y-axis) was much lower than for the rest.

Table 3.2: Microsatellite profiles of leaf samples (n=38) collected from different branch positions in four *E. camaldulensis* trees.

Tree ID-Replicate	Branch position	*Eg99*	*Eg98*	*Eg16*	*Eg91*	*Eg65*	*Eg67*	*Eg84*	*Eg96*
Ec 3278-1	Lower	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-2	Mid	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-3	Upper	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-4	Mid	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-5	Lower	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-6	Lower	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-7	Upper	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-8	Mid	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-9	Mid	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3278-10	Upper	191/194	172/181	237/243	140/144	234/249	158/160	106/120	274/283
Ec 3328-1	Lower	191/197	169/175	240/243	Null/Null?	234/249	Null/160	106/128	277/279
Ec 3328-2	Lower	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-3	Mid	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-4	Upper	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-6	Upper	191/197	169/175	240/243	134/140	234/249	Null/160	106/128	277/279
Ec 3328-7	Mid	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-8	Mid	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-9	Upper	191/197	169/175	240/243	134/140	234/249	154/160	106/128	277/279
Ec 3328-10	Mid	191/197	169/175	240/243	134/140	234/249	Null/160	106/128	277/279
Ec A-1	Lower	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-2	Lower	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-3	Mid	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-4	Upper	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-5	Mid	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-6	Low	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-7	Upper	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-8	Mid	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-9	Low	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec A-10	Upper	194/194	178/184	237/240	140/145	234/234	158/186	106/120	274/292
Ec 3311-1	Lower	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-2	Lower	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-3	Mid	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-4	Mid/Upper	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-5	Upper	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-6	Mid	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-7	Low	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280
Ec 3311-8	Upper	197/197	175/175	237/237	138/138	255/255	160/180	106/114	280/280

Table 3.3: Microsatellite profiles of leaf samples (n=37) collected from different branch positions in four *E. leucoxylon* trees.

Tree ID-Replicate	Branch position	*El14*	*El29*	*El01*	*El07*	*El28*	*El18*	*El13*	*El16*
El001-1	Upper	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-2	Mid	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-3	Mid	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-4	Mid	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-5	Low	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-6	Mid	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-7	Low	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El001-8	Upper	184/192	270/276	375/387	125/153	222/222	299/311	192/200	230/232
El002-1	Low	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-2	Low	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-3	Upper	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-4	Low	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-5	Upper	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-6	Mid	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-7	Mid	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-8	Mid	168/184	268/268	358/375	121/127	222/222	299/309	200/200	228/232
El002-9	Low	168/184	268/268	358/375	**122/128**	222/222	299/309	200/200	228/232
El003-1	Mid	168/170	274/274	375/375	145/151	222/222	295/297	**199/199**	232/234
El003-2	Mid	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-3	Mid	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-4	Mid	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-5	Upper	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-6	Low	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-7	Mid	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-8	Low	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El003-9	Mid	168/170	274/274	375/375	145/151	222/222	295/297	200/200	232/234
El005-1	Low	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-2	Mid	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-3	Upper	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-4	Mid	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-5	Mid	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-6	Low	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-7	Mid	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-8	Mid	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
El005-9	Low	168/182	268/270	358/375	123/125	208/222	301/301	182/200	234/236
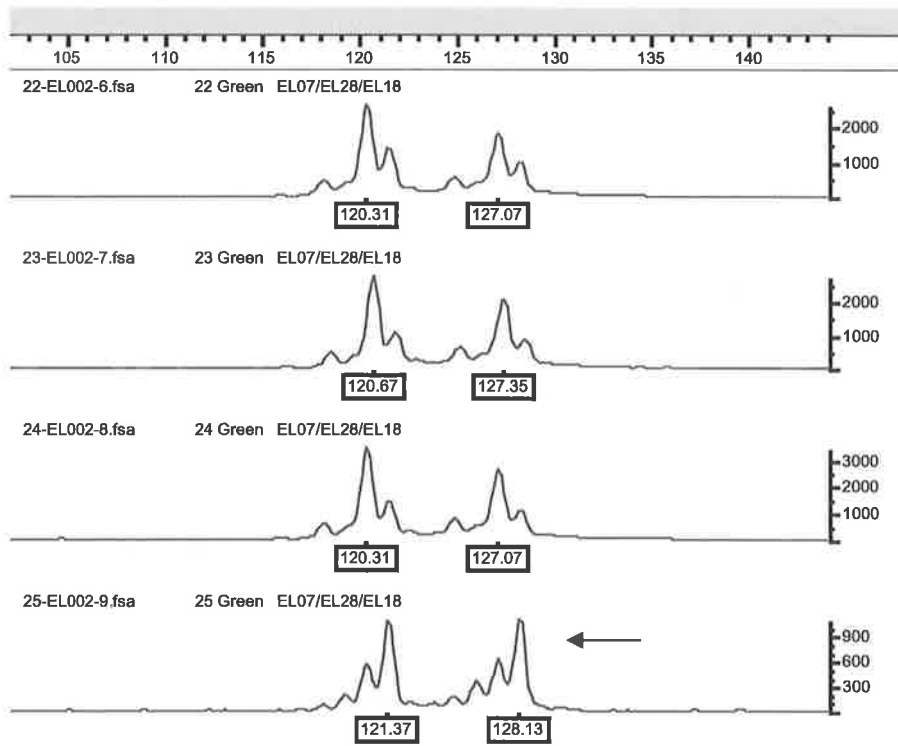
Figure 3.1: Electrophoretic profile of *E. leucoxylon* samples at locus *El07* showing inconsistency in allele shape for sample EL002-9.
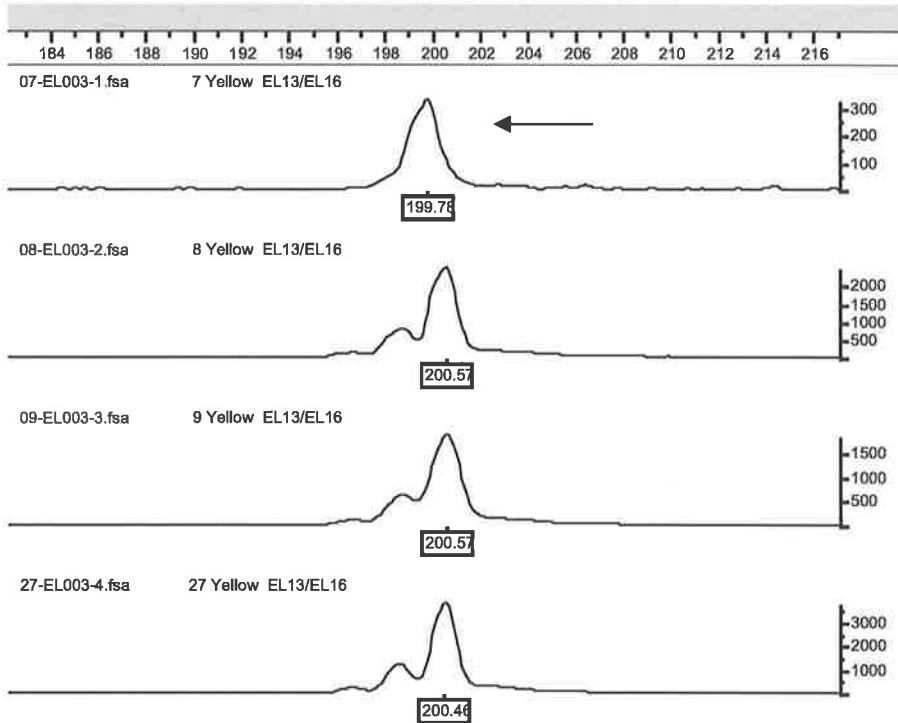
Figure 3.2: Electrophoretic profile of *E. leucoxylon* samples at locus *El13* showing inconsistency in allele shape for sample EL003-1.

3.4 Discussion

3.4.1 Somatic variation in microsatellite profiles

Despite microsatellite loci having a high mutation rate in comparison to other markers, no stepwise mutations were detected amongst 16 nuclear microsatellite loci in the somatic tissue of two eucalypt species. However, there were potentially two other types of mutations present. In two cases, a single base pair shift in microsatellite allele sizes appeared present at loci *El07* and *El13*, and for both samples it appeared that the 1bp shift occurred at both alleles. It would seem unlikely that a 1 base pair insertion/deletion mutation would occur simultaneously in both alleles – instead, it would be more likely that the 1bp shift visualised was due to errors during the PCR or electrophoresis procedure (e.g. stuttering). More extensive sampling may have indicated whether the shift was "real" or alternatively, sequencing both alleles would have confirmed whether a 1bp insertion/deletion was present. Both of these approaches would have been time-consuming when it is most likely that the differences observed were due to PCR error.

At another locus, *Eg67*, three samples produced a PCR profile that appeared to contain a "null" allele. Subsequent work involving this locus showed that its PCR amplification was unpredictable and that null alleles appeared to be common. Presumably, the *Eg67* primers are only marginally compatible with the flanking sequence of the target microsatellite in *E. camaldulensis* (the primers were transferred from another Eucalypt species, *E. globulus*) and that a small mutation in the flanking sequence (e.g. close to the 3' end of the primer site) is enough to prevent the primers binding. The failure to amplify one of the alleles is also potentially due to problems encountered during the PCR process (e.g. low quality DNA) and not necessarily due to a mutation in the primer binding site. One of the first steps to resolve this issue would be to re-extract DNA from the leaf samples and PCR amplify them again. If the null allele subsequently appeared to be "real", the next step would be to re-design primers from the microsatellite flanking sequence of the original *E. globulus* clone (from which the *Eg67* primers were designed) that were external to the current primers. The new primers would then amplify a product that encompasses the *Eg67* primer sequence. This could be sequenced and leaf samples that contained the null allele and samples that amplified the correct allele could be compared to detect whether a mutation was present.

However, for the purposes of this thesis I did not go down this path, as *Eg67* was not to be included in any mating system or parentage analyses.

3.4.2   Power to detect microsatellite mutations in somatic tissue

Meiotic mutation rates for microsatellite loci in plants have been estimated to fall within the range of 10^{-2}-10^{-5} for nuclear-encoded loci with tri and dinucleotide repeats (Kovalchuk *et al.* 2000; Udupa & Baum 2001). It is unclear whether mitotic mutation rates are of the same order, but two studies have reported somatic mutation rates to be in the order of 10^{-4} per generation (O'Connell & Ritland 2004) and 10^{-6} to 10^{-7} per mitosis (Cloutier *et al.* 2003). In this study, I sampled somatic tissue from a relatively small number of positions in the canopy and from a small number of trees, in order to replicate the sampling design I intend to use for future mating system studies. It is possible that the limited sampling design may have affected the ability to detect somatic mutations if the rate of somatic mutations is low.

O'Connell & Ritland (2004) estimate the rate of somatic mutations at microsatellite loci from the equation $U=m/NLK$, where U is the per-generation mutation rate, m is the number of mutations observed, N is the number of tissue samples per tree, L is the number of trees sampled and K is the number of loci sampled (see O'Connell & Ritland (2004) for derivation of equation). The estimator makes two major assumptions: 1) that trees of average mature age have been sampled and 2) that new mutations are identified in an unbiased manner (that is, somatic tissue should be collected from points far apart enough in the tree to reduce the probability of sampling the same mutation more than once, assuming the mutant sector is sufficiently small). The *E. camaldulensis* and *E. leucoxylon* trees sampled in this study were large, mature trees of approximately similar age (though EL001 was smaller than the other trees and potentially younger). Leaf tissue was sampled from up to ten different, widely-spaced branches in the canopy and would represent an unbiased sample unless a mutation occurred very low down on the tree and all branches sampled were mutant.

Therefore, to estimate the power to detect somatic mutations in this study, the above equation can be solved for L, the number of trees that need to be sampled to detect one mutation. For each species, eight microsatellite loci were sampled (K), an average of

nine leaf samples were assayed per tree (N) and we would like to detect one mutation (m). If we assume the per generation mitotic mutation rate to be 10^{-4} (as estimated in O'Connell & Ritland 2004), $L=1/8*9*0.0001=139$ trees. If we assume a faster mutation rate of 10^{-3}, $L=14$. A slower mutation rate of 10^{-6} would require 13900 trees to be sampled to detect one mutation. In all cases, more trees needed to be sampled to detect a single mutation at microsatellite loci than were sampled in this study.

However, O'Connell & Ritland's (2004) estimator of mutation rate is based on a per generation mutation rate, rather than a per cell division mutation rate. Intuitively, we would expect to have a higher probability of detecting a somatic mutation in tissue collected from the top of a tree than in tissue collected from the base, simply due to the number of mitotic divisions that have occurred in between. Estimating mitotic mutation rates by calculating the number of cell divisions leading to a new mutation is problematic, as it requires several assumptions (O'Connell & Ritland 2004). One way to estimate the number of cell divisions per unit growth is to estimate the average size of vegetative cells and then calculate the number of cells in, for example, a metre of growth. The assumption is then that cells are a constant size throughout the plant. Cloutier *et al.* (2003) have used this approach and estimate that between 47 170 and 14 205 cell divisions occur per 1 metre unit of growth in *Pinus strobus*, based on the average length of the smallest and largest cells within shoot tips. Thus, they estimate that somatic mutation rates for nuclear microsatellite loci of between 1.38×10^{-7} and 4.59×10^{-7} mutations per cell division.

Our ability to detect somatic mutations in tissues sampled from a tree will also depend on the fate of the apical initial cell in which the mutation arose. In higher plants, the apical initial cells divide to form three cell layers (tunica-corpus meristematic organisation) that then give rise to plant tissues and organs. In most angiosperms, the L1 layer cells produce the epidermis, the L2 layer cells produce the subepidermal cells of the stem and lateral organs (e.g. mesophyll of leaves) and the L3 cells form the pith of the stem and interior tissues of organs (e.g. phloem and xylem) (Haecker & Laux 2001). In the case of leaf tissue, mesophyll cells are the most abundant and therefore would contribute the most DNA to a genomic DNA extraction. Therefore, we would only be most likely to detect a somatic mutation if it had occurred in an apical cell that ended up in the L2 layer of the meristem. In addition, the mutation would only be

heritable if the mutation occurred in a cell in the L2 layer, as it is the L2 layer cells that give rise to the gametes (Gill *et al.* 1995).

Theoretical models also predict that somatic mutations will be relatively hard to detect. For example, a theoretical treatment of somatic mutations in clonal organisms predicted that in a colony of 10^6 cells with a mutation rate of 10^{-7}, most colonies (95.1%) would have no mutants (Luria & Delbruck 1943, cited in Gill *et al.* 1995). Likewise, Antolin & Strobeck (1985) found that only if mutation rates were high (10^{-3}) and strong selection favoured the survival of the mutant cells would we be able to detect mutations in a significant proportion (30%) of tissues. Otherwise, less than 5% mutant cells were detected when mutation rates were lower (10^{-4} to 10^{-6}). A similar finding was reported by Pineda-Krch & Lehtila (2002) based on the fitness benefit of the mutation – only when mutation fitness was high would we be able to detect a mutation in >30% of tissues (maximum value was 45% at the highest mutation fitness). While microsatellites have an apparently high mutation rate they have long been considered to be selectively neutral (Awadalla & Ritland 1997; Schlotterer & Wiehe 1999). Therefore, it is unlikely that mutations at microsatellite loci will be positively selected for *per se*. The exception to this would be if the microsatellite had functional significance or if it was found in close linkage with other selected loci (e.g. Slatkin 1995a; Li *et al.* 2002). Consequently, a cell lineage containing a microsatellite mutation would not be likely to proliferate and therefore would only be present in a very small proportion of the entire plant. As such, it is likely that a more exhaustive sampling of plant tissues than conducted in the present study would be required to increase the possibility of detecting somatic mutations of microsatellite loci.

Finally, microsatellite mutation rates are known to vary for individual loci, depending on the structure of the microsatellite region – for example, the number of repeats, the sequence of the repeat motif or the flanking sequence and interruptions in the microsatellite may all influence the rate at which a microsatellite locus may mutate (Ellegren 2000). To date, O'Connell & Ritland (2004) are the only researchers to directly detect a single somatic mutation at a microsatellite locus. Interestingly, the locus at which the mutation occurred was one of the most variable in their target species. The $(AC)_n$ repeat contained from 20 to 59 repeat units and 27 alleles were detected in 80 individuals. The microsatellite loci used in this study did not show such

extraordinary levels of variation. Allele number of *E. camaldulensis* microsatellites ranged from 5 to 11 in a survey of 63 individuals and the number of alleles at *E. leucoxylon* microsatellite loci ranged from 8 to 20 amongst 68 individuals (Chapter 2). Again, it appears that the ability to detect microsatellite mutations in somatic tissue may be further limited to loci with very high mutation rates.

3.5 Conclusions

A number of lines of evidence suggest that genetic mutations may be difficult to detect in somatic tissues, except under conditions of extremely high mutation rates and strong levels of selection for the survival of mutant cell lineages. This study utilised a sampling design similar to what would be used in a mating system study, where plant tissue was collected from up to 10 major tree branches throughout the tree canopy. Under this design no step-wise microsatellite mutations were detected, suggesting that somatic mutation rates of microsatellites are not sufficiently great in *E. camaldulensis* or *E. leucoxylon* to interfere with the assumptions required for mating system and parentage studies to be conducted in this research project. In general though, this does not preclude the presence of within-tree microsatellite mutations in these or other long-lived species, as a more extensive sampling design (more trees, more sampling positions, more loci) may potentially uncover microsatellite mutations. As somatic microsatellite mutations have been observed previously, researchers should be aware that somatic mutations are a potential source of error when somatic and reproductive tissue are compared in mating system and parentage analyses. Performing a pilot study similar to the study conducted here could easily assess the scale of the problem.

Chapter 4   A survey of the physical and genetic characteristics of *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees

4.1   Introduction

Temperate eucalypt woodlands were once common throughout southern Australia, providing a transition zone between the forested landscapes of the relatively wet coastal margins and the drier shrublands and grasslands of the interior. The relatively fertile soils on which eucalypt woodlands occur attracted the attention of European settlers and extensive clearing for cropland and grazing occurred from an early stage and continues today (Yates & Hobbs 1997; Yates *et al.* 2000; Paton *et al.* 2004b). In South Australia, the MLR contains the largest remnants of eucalypt woodland communities in the state and is recognised as a National Biodiversity Hotspot. However, in some areas of the MLR less than 2% of the native vegetation remains, while, overall, greater than 90% of the woodland vegetation has been lost from the MLR region (Paton *et al.* 2004b) (Figure 4.1). The remaining native woodland vegetation in the MLR is highly fragmented and persists as widely spaced patches of more-or-less intact woodland habitat (only a small proportion of which is protected in conservation reserves) or as scattered trees and clumps of trees that were not removed when the rest of the woodland vegetation was cleared (Paton *et al.* 1999).

(Source: http://www.atlas.sa.gov.au)
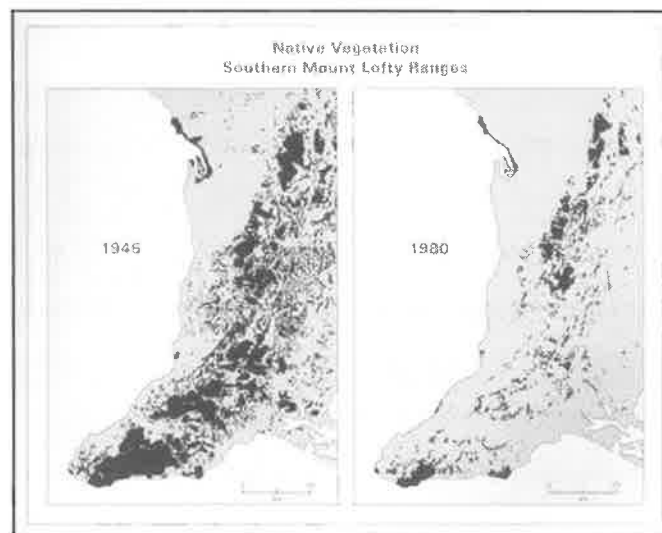
Figure 4.1: Native vegetation change in the Southern Mount Lofty Ranges from 1945 to 1980.

Indeed, in the agricultural regions of south-eastern Australia, scattered paddock trees form a substantial component of the remnant native vegetation, and in many cases represent the last remnants of particular plant communities. For example, in a study of woodland eucalypt communities on the south-west slopes of NSW, Gibbons & Boak (2002) found that for both Blakely's Red Gum/Yellow Box communities and White Box/Red Stringybark communities, 54% of remnant vegetation occurred in patches <1ha in size (which includes paddock trees and small clumps of trees). Similarly, in south-east South Australia, 100% of Pink Gum (*E. fasiculosa*) and Blue Gum (*E. leucoxylon*) Low Woodlands exist as paddock trees or patches <1ha in size (Carruthers *et al.* 2004). While previous research has focused on the dynamics of remnant woodland vegetation patches (e.g. Prober & Brown 1994; Hobbs 2001; Major *et al.* 2001), scant information exists on the abundance or ecological status of scattered woodland trees in the agricultural regions of southern Australia. With the current rate of decline of paddock trees and patches of trees, it is estimated that these trees could be lost from some regions of Australia in as little as 40 years (Ozolins *et al.* 2001; Gibbons & Boak 2002; Carruthers *et al.* 2004).

Because of the vital role paddock trees play in maintaining ecological and agricultural productivity, scattered trees have been referred to as "keystone" structures (analogous to the concept of "keystone species"; Manning *et al.* 2006). That is, scattered trees have a disproportionate effect on the ecosystem relative to the small area they occupy, the low biomass of any given tree and the collective low density of scattered trees in the landscape. Scattered trees provide habitat and resources for a number of plant, vertebrate and invertebrate species, they are important in maintaining soil nutrient and hydrological properties, as well as providing "stepping stones" for the movement of animals and genes across the landscape (reviewed in Chapter 1). In addition, paddock trees may represent a store of genetic information and may contribute to the long-term persistence of tree populations through pollen- and seed-mediated gene flow. However, little is known about the genetic diversity that may be extant in these trees or the distribution of genetic diversity across the population. The impacts of habitat clearance (especially the reduction of plant density and the resultant spatial distribution of plants) on the pollination and mating system of isolated *Eucalyptus* trees are unknown. It is not clear whether pollinator populations and pollination services (and thus dispersal of genes via pollen) can be maintained in such a highly modified system.

Eucalyptus camaldulensis (River Red Gum) and *E. leucoxylon* (South Australian Blue Gum) are both woodland eucalypts that were once widespread and abundant throughout the southern MLR, but that now predominantly exist as scattered trees. In this chapter, I report the results of a survey of the physical, demographic and genetic characteristics of a sample of scattered trees of each species. Firstly, I explore the spatial distribution of these trees in the landscape and whether their distribution may be associated with any particular landscape features. Secondly, I surveyed a range of physical features of the trees to characterise the age structure and health of scattered tree populations. I also report the results of a genetic survey of these trees that was conducted to assess the potential contribution of remnant trees to the maintenance of genetic diversity of each species.

4.2 Methods

4.2.1 Study species and location

Eucalyptus camaldulensis forms woodlands in the valleys of the MLR and grows best on fertile, slightly acid soils. The species has a relatively high soil moisture requirement, thus is often the dominant large tree species found along streams and rivers. As the original understorey was relatively open, river red gum woodland came under grazing pressure from early settlement days (Boomsma & Lewis 1980). *Eucalyptus leucoxylon* is one of the most common woodland tree species in the MLR, forming significant woodland associations with at least eight other eucalypt species (Boomsma & Lewis 1980). It is common on the lower slopes and valley floors of the MLR, indicating a high soil moisture and nutrient requirement.

Eucalyptus camaldulensis paddock trees were located on private landholdings near Tungkillo (323700E/6144400N) and *E. leucoxylon* trees near Flaxley (301500E/6109400N) in the MLR, South Australia (Table 4.1). At both sites, extensive vegetation clearance has occurred for agriculture. At the drier Tungkillo site (annual rainfall 640mm) land use is predominantly sheep and cattle grazing while at Flaxley (annual rainfall 810mm) the predominant land use is dairy cattle grazing on improved pasture, though sheep grazing is also prevalent on one property (Table 4.1). The area comprises gently undulating hills (elevation 360-440m at Tungkillo and 360-400m at Flaxley) and is intersected by numerous small creeks and drainage lines.

Table 4.1: Land use characteristics of properties on which scattered trees were located.

Property	Location	Land Use	Remnant Veg (>1ha) present?	Scattered trees	Landcare activities
E. camaldulensis					
"Roskhill" (N. Skinner)	316778E 6140778N	Primarily sheep grazing, some cattle	Yes, moderately large patches, good condition	Moderately high density	Remnant veg patches fenced
M. Collins	321946E 6141283N	Sheep grazing	No	Moderate density along creeklines, low density away	Fenced reveg along creeklines. Some remnant trees included in reveg patches
R. Guthrie	321138E 6140605N	Primarily sheep grazing, some cattle	No	Mostly low density, one patch of high density trees along creekline	Fenced reveg on upper slopes. Few remnant trees included in reveg patches
E. leucoxylon					
"Battunga" (A. Kebbell)	300374E 6109508N	Sheep and cattle grazing, one area of cultivation	Yes, small patches, variable condition	Low density	Fenced reveg along creeklines. Some remnant trees included in reveg patches
Flaxley Dairy Research Centre	301156E 6110187N	Dairy cattle grazing (fertilised and irrigated paddocks)	Yes, small patches, opportunistic grazing	Low density	Creeklines fenced. No reveg activities
D. Kuchel	300919E 6108628N	Dairy cattle grazing	Yes, small patches, currently grazed	High density in remnant patches, low density in other areas	None

Small patches of remnant vegetation are present at both field sites and indicate previous vegetation associations. At Tungkillo, *E. camaldulensis* scattered trees were once part of a Red Gum (*E. camaldulensis*)/Blue Gum (*E. leucoxylon*)/Allocasuarina (*A. verticillata*) woodland association. At Flaxley, *E. leucoxylon* scattered trees were found in association with Pink Gum (*E. fasiculosa*)/Stringybark (*E. baxteri*) woodland. At both sites, paddock trees are now the predominant remnant vegetation type. All trees included in this study occurred over native or introduced pasture grasses and were exposed to land use activities including grazing, stock camping, irrigation (Flaxley Research Centre) or cultivation (Battunga) (Table 4.1).

4.2.2   Scattered tree demographic and physical characteristics

Thirty paddock trees each of *E. camaldulensis* and *E. leucoxylon* were surveyed for a range of physical and demographic characteristics in December 2003. At both locations, tree clearance has not been uniform, such that in some areas local tree density is moderately high, while in other areas tree density is extremely low. In order to get a sample of trees that represented the range of demographic scenarios present at each site, I initially selected paddock trees to study based upon their "degree of isolation". This assessment was based upon the distance of a paddock tree to it's nearest neighbour and/or conspecific (e.g. 0-50m, 51-100m, etc) and a qualitative assessment of local tree density (e.g. low (<1 tree/ha), medium (2-5 trees/ha), high (6+ trees/ha)). Trees were located across an area of approximately 450ha at Tungkillo and 420ha at Flaxley.

Paddock trees were identified in the field and then located on orthorectified aerial photographs. Distances between trees and their nearest neighbour (*distance to NN*) and nearest conspecific (*distance to NC*) were measured from aerial photos in Arcview (v3.2a). "Local" tree density was calculated for a sub-sample of trees using quadrats overlaid on the aerial photographs. Quadrats were centred on a focal tree and the number of trees (of any species) and the number of conspecifics (same species) within the quadrat were counted. The focal trees chosen were dispersed across the study sites as much as possible. *Eucalyptus camaldulensis* local tree density was estimated using 500 x 500m quadrats, but smaller quadrats (200 x 200m) were used to estimate density of *E. leucoxylon* paddock trees, as the larger quadrats overlapped roadside vegetation for which tree identity and tree density had not been determined in the field. Mean

paddock tree density (number of trees per hectare) for each location was then calculated from these estimates.

The physical distribution of trees was assessed in the field by noting their location on the slope (lower, mid, upper), the direction of the slope and the degree of the slope (gentle, steep). The location of other landscape features was also noted (e.g. proximity to creeklines). The use of paddock trees by livestock was assessed qualitatively by observing the condition of the ground beneath the tree canopy, including the relative abundance of grass cover, leaf litter and bare ground, the degree of soil compaction and the amount of stock faeces. Tree use was categorised as low, medium or high use, relative to the range of conditions observed at each site.

Trees were also measured for a range of physical characteristics including diameter at breast height (*DBH*, stem circumference was measured at 1.3m from the soil surface and diameter was calculated from the equation $C=\pi d$, in centimetres); tree height (*h*, visually estimated to the nearest metre) and canopy width (measured at the maximum canopy width (*w1*) and at 90° to the maximum canopy width (w2), in metres). Canopy area was calculated assuming the canopies were circular in shape (more-or-less true for paddock trees) by determining the average radius of the canopy from *w1* and *w2* and using the formula $\pi.r^2$ (in metre^2). Canopy volume was calculated using the formula $\pi.h.w1.w2/6$ (in metre^3) (Hogbin *et al.* 1998). Stem volume was calculated from West's (2003) average function, $V=0.281.DBH^{1.91}.h^{1.02}$ where *V* is the total underbark stem volume from the ground to the tip (in metre^3).

I compared the size distribution of a sample of low to medium density paddock trees of each species, with that of trees found in "natural" density arrays. "Natural" density arrays refer to trees found in intact remnant vegetation and also paddock trees in clumps, where trees appear to be at their natural density but where the understorey has been removed through grazing. For *E. camaldulensis*, "natural" density trees were sampled from a 16ha patch of intact remnant vegetation located adjacent to paddock trees at Roskhill (Table 4.1). *Eucalyptus leucoxylon* trees were sampled from a 40ha remnant vegetation patch located approximately 1.5km to the south of D. Kuchel's property, as this was the closest large patch of relatively undisturbed vegetation to the

properties on which paddock trees were sampled. In both cases, I assumed that trees in remnant vegetation experienced the same environmental conditions (e.g. similar topography, soils, climactic conditions) as the paddock trees sampled. An additional three trees from each species were sampled from the intact remnant vegetation patches.

In eucalypts, it is generally accepted that tree DBH is related to tree age, though the precise relationship may vary depending on stand characteristics, the canopy position of trees, microsite conditions and genetic differences (Alcorn *et al.* 2001). Since generalized age-size relationships are unknown for either *E. camaldulensis* or *E. leucoxylon*, I estimated the age structure of paddock tree populations based on a diameter growth rate of 5mm.yr^{-1}. This estimate comes from the large dataset of Stoneman *et al.* (1997) of actual tree growth measurements and estimations from tree ring analyses of *E. marginata*, a dominant forest tree from south-western Australia. I chose to apply this growth rate estimate as Stoneman *et al.*'s (1997) dataset is the largest published for any eucalypt species in Australia, it relates to a canopy dominant species and climactic conditions are broadly similar between southern WA and the MLR.

I also assessed tree condition using a number of measures. The extent of intact canopy was assessed by estimating the proportion of the canopy that contained living leaf material. The volume of intact canopy was then calculated by multiplying the proportion of intact canopy by the canopy volume as calculated above. The proportion of canopy that contained epicormic growth and the overall density of foliage (%) were also recorded. I also surveyed for the presence of eucalypt seedlings (germinants and juveniles) within a 20m radius of each tree, though no seedlings were located during the study period.

4.2.3   Genetic analyses

Genomic DNA was extracted from leaf material and genotyped at seven (*E. camaldulensis*) or eight (*E. leucoxylon*) microsatellite loci using conditions described in Chapter 2. Descriptive population genetic parameters (N_a, H_e, H_o, F_{is}) were estimated from genetic data of adult trees in GenAlEx v5.8 (Peakall & Smouse 2005). I estimated the mean relatedness of trees using Queller & Goodnight's (1989) estimator R,

calculated in GenAlEx. A relatedness estimate of $R=0$ indicates unrelated individuals, $R=0.25$ indicates half-sibs and $R=0.5$ indicates full sibs. The program Identity (Wagner & Sefc 1999) was used to check for identical genotypes. Spatial autocorrelation of genotypes was assessed at the fine-scale (0-250m) and on a landscape scale (*E. camaldulensis* 0-5km; *E. leucoxylon* 0-2.5km). Spatial autocorrelation (r) analyses were also performed in the program GenAlEx. Error terms for r were determined from 1000 bootstrap pseudoreplicates of mean r and significance of r was determined by comparing observed r to the mean of 999 permutations of r.

4.3   Results

4.3.1   Density and distribution of scattered trees

At the landscape scale, the spatial distribution of paddock trees differed between the two eucalypt species. At Tungkillo, *E. camaldulensis* is the most common eucalypt species occurring as paddock trees, with very little intermixing with other eucalypt species (Figure 4.2). However, at Flaxley, *E. leucoxylon* isolated trees mix with *E. fasiculosa* (Pink Gum) or *E. baxteri* (Brown Stringybark) on the upper, drier slopes with thinner soils and with *E. camaldulensis* near creeklines (Figure 4.3). *Eucalyptus camaldulensis* scattered trees overall had a more uniform, continuous distribution across the landscape than *E. leucoxylon* trees. *Eucalyptus leucoxylon* scattered trees tended to occur in patches of 5-6 individuals, interspersed by patches of scattered pink gum or stringybark trees and, thus, on a landscape scale, occurred at much lower densities than *E. camaldulensis*.

Local paddock tree density (estimated from quadrats) was higher at the *E. leucoxylon* site when all tree species were included (average 3.4 trees/ha vs. 0.8 trees/ha) (Table 4.2). Tree clearance at Tungkillo has been more uniform with little variation in local tree density (all species) for most focal trees. At Flaxley, however, local tree density was variable between focal trees. Overall though, local conspecific tree density was approximately 1 tree/ha for *E. camaldulensis* paddock trees, and 0.86 trees/ha for *E. leucoxylon*.

Figure 4.2: Aerial photograph showing distribution of *E. camaldulensis* paddock trees at Tungkillo, MLR.
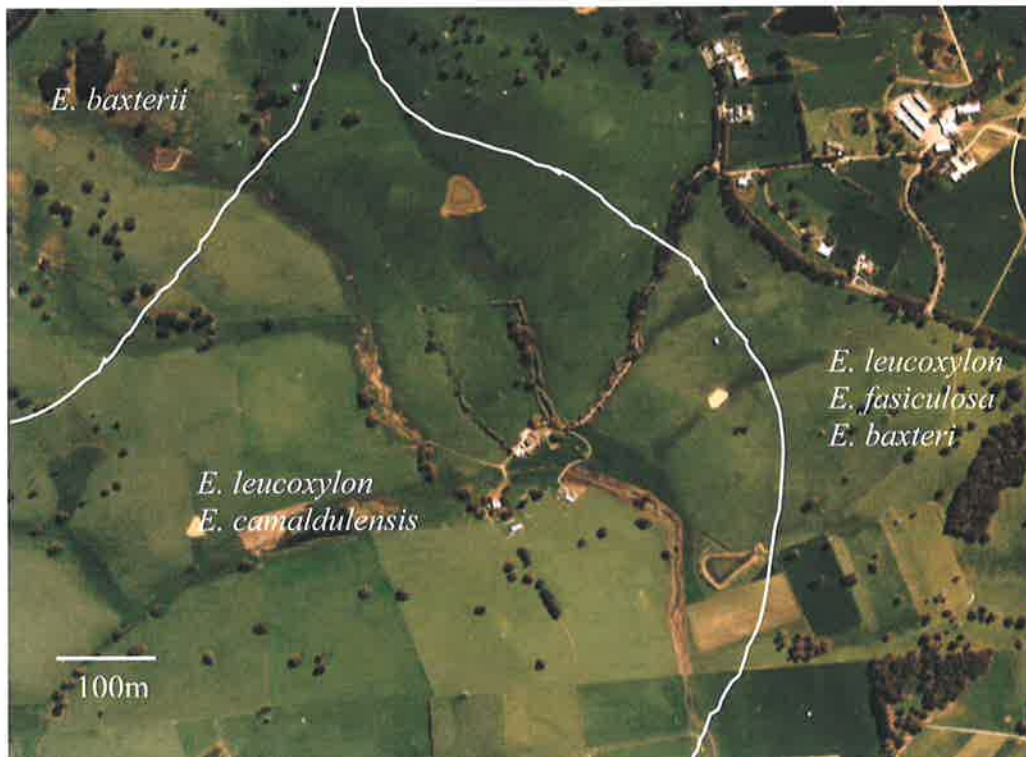
Figure 4.3: Aerial photograph showing distribution of eucalypt paddock trees at Flaxley, MLR.

At this site *E. leucoxylon* paddock trees intersperse with *E. fasiculosa*, *E. baxteri* and *E. camaldulensis* trees.

Table 4.2 : Local tree density (no. trees/ha) and mean distance to nearest neighbours for scattered trees of *E. camaldulensis* and *E. leucoxylon* (± S.E.).

Focal tree	Local density paddock trees[a] (trees/ha)	Local density conspecifics[b] (trees/ha)
E. camaldulensis		
Ec3348	0.80	0.80
Ec3333	0.64	0.64
Ec3338	0.26	0.26
Ec3293	3.00	2.84
Ec3315	1.36	1.36
Ec3301	0.28	0.28
Mean (trees/ha)	**1.06 ± 0.4**	**1.03 ± 0.4**
Mean *distanceNN* (m)	**62 ± 11**	**65 ± 11**
E. leucoxylon		
El5601	0.36	0.16
El5613	2.25	1.0
El5607	5.75	1.0
El6493	4.5	1.25
El5612	6.0	1.5
El5622	1.25	0.25
Mean (trees/ha)	**3.35 ± 1.0**	**0.86 ± 0.2**
Mean *distanceNN* (m)	**35 ± 7**	**72 ± 15**

[a] All tree species included; [b] Conspecifics only.

Table 4.3: The number of surveyed scattered *E. camaldulensis* and *E. leucoxylon* trees located at different slope positions and mean nearest neighbour distances (± S.E.).

Distance to NN = Distance to nearest neighbour of any tree species
Distance to NC = Distance to nearest neighbour conspecific

	Creek line	Lower	Mid	Upper
E. camaldulensis				
No. trees	5	3	17	5
Distance to NN (m)	17 ± 11	46 ± 24	70 ± 15	87 ± 29
Distance to NC (m)	17 ± 11	61 ± 13	73 ± 16	87 ± 29
E. leucoxylon				
No. trees	2	1	18	9
Distance to NN (m)	35 ± 3	10	36 ± 42	37 ± 41
Distance to NC (m)	93 ± 84	10	83 ± 97	52 ± 60

The differences in scattered tree distribution between the two species were also reflected in the average distances to nearest neighbours and conspecifics. At Tungkillo, *E. camaldulensis* was the predominant species represented as scattered trees, and, consequently, the distance to the nearest neighbour was frequently the distance to the nearest conspecific. For *E. camaldulensis*, the average distance to an individual of the same species was approximately 65m (Table 4.2). Scattered tree density (all species included) was higher at Flaxley and this was also reflected in the difference in distance to nearest neighbour and conspecifics for *E. leucoxylon* scattered trees. *Eucalyptus leucoxylon* trees were located close (mean=37m) to other trees but distant to other *E. leucoxylon* individuals (mean=72m). Overall, the average distance to conspecifics was similar for both species.

Eucalyptus camaldulensis and *E. leucoxylon* scattered trees also differed with respect to landscape features (i.e. slope position, proximity to creek lines) (Table 4.3). *Eucalyptus camaldulensis* had a more uniform distribution across the landscape than *E. leucoxylon*, with *E. leucoxylon* primarily being restricted to mid and upper slope positions. As a species *E. camaldulensis* is most frequently associated with water features and at Tungkillo a significant proportion of trees were located along creek lines. In addition, *E. camaldulensis* trees associated with creek lines had the smallest nearest neighbour distances suggesting that these areas have been less heavily cleared. At Flaxley, few *E. leucoxylon* trees were associated with creek lines as presumably these sites were more frequently occupied by *E. camaldulensis*. In both species, few trees were found on the lower areas of slopes. The majority of trees in both species occurred mid-slope, while *E. leucoxylon* were also frequently located on upper slopes. In *E. camaldulensis* there was a trend for increasing tree isolation at upper slope positions, but it was hard to detect a trend for *E. leucoxylon* due to small sample sizes and greater variance in estimates of isolation distances. A comparison of isolation distances for mid and upper slope trees suggests that *E. leucoxylon* has been less heavily cleared on upper slopes.

4.3.2   Tree dimensions and size distribution

Paddock trees of both species of eucalypts were of large girth and had spreading canopies (Table 4.4). On average, *E. leucoxylon* scattered trees tended to have a slimmer trunk, were slightly taller and less spreading than *E. camaldulensis* trees.

Though *E. camaldulensis* was on average slightly smaller in height than *E. leucoxylon*, *E. camaldulensis* trees supported a greater volume of canopy, reflected in the ratio of tree height to canopy volume (1:154 for *E. leucoxylon* and 1:262 for *E. camaldulensis*). Average stem volume was approximately three times greater in *E. camaldulensis* trees than *E. leucoxylon*.

When compared to the dimensions of trees found at "natural" densities, paddock trees of both species had significantly greater *DBH*, height, canopy spread, canopy volume, and stem volume (Table 4.4). Indeed, the majority of paddock trees sampled of both species had a *DBH* greater than 100cm (75% of *E. camaldulensis* trees and 50% of *E. leucoxylon* trees), and very few trees surveyed had a girth less than 50cm (Figure 4.4). The skewed distribution of trees towards larger size classes may have resulted from the pattern of vegetation clearance (e.g. farmers leaving large individuals when the rest of the vegetation was cleared) or potentially these trees may have subsequently obtained large size when released from density dependent competition following vegetation clearance. For paddock trees in high to medium density situations (1-50m distance to nearest neighbour), tree size is correlated strongly with distance to nearest neighbour in both species (Table 4.5), with the exception of height in *E. leucoxylon*, indicating a degree of density dependent growth.

Applying a diameter growth rate of 5mm/yr (Stoneman *et al.* 1997), *E. camaldulensis* trees range from 45–573 years of age (mean = 293 ± S.E. 130 years) and *E. leucoxylon* trees range from 71-351 years (mean = 190 ± S.E. 73 years). Whilst it may be problematic estimating the age of trees from size data, it is nonetheless clear that the population of paddock trees sampled in this study are heavily skewed towards older individuals and young trees are under-represented. In addition, no seedlings or saplings of either eucalypt species were observed during the survey period, indicating a complete absence of recruitment in the paddock environment for an extended period of time.

Table 4.4: Mean (± S.E.) tree dimensions of *E. camaldulensis* and *E. leucoxylon* trees at "natural" density and paddock trees.

Superscripts represent significant differences in mean tree dimensions of paddock trees compared to trees at "natural" density, as determined by a t-test. Significance levels: *** $p<0.001$; ** $p<0.01$

Measurement	*E. camaldulensis*		*E. leucoxylon*	
Vegetation type	Natural	Paddock	Natural	Paddock
n	10	23	9	22
DBH (cm)	73 ± 13	169 ± 11 ***	44 ± 4	108 ± 7 ***
Height (m)	14 ± 1.1	18 ± 0.6 ***	14 ± 1.1	20 ± 0.7 ***
Canopy area (m²)	42 ± 7	68 ± 3 **	36 ± 3	53 ± 2 ***
Canopy volume (m³)	$1\,807 \pm 579$	4716 ± 408 ***	1181 ± 288	3073 ± 303 ***
Stem volume (m³)	214 ± 70	1135 ± 171 **	66 ± 14	488 ± 62 ***

Table 4.5: Pearson's correlation coefficients (*r*) of tree size measurements with distance to nearest neighbour for trees located 1-50m from their nearest neighbour.

All variables were normally distributed with the exception of *distance to NN*, which was square root transformed to satisfy assumptions of normality. Significance levels: *** $p<0.001$; ** $p<0.01$

	n	*DBH*	Height	Canopy area	Canopy volume
E. camaldulensis	15	0.789 ***	0.536 ***	0.468 **	0.517 **
E. leucoxylon	21	0.676 ***	0.121 n.s.	0.335 **	0.376 **
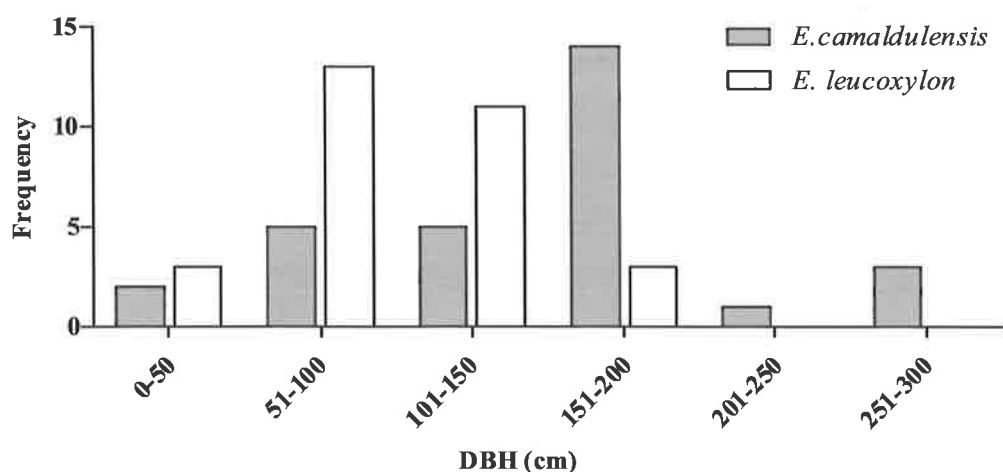
Figure 4.4: Histogram of DBH (cm) of *E. camaldulensis* and *E. leucoxylon* paddock trees.

4.3.3 Tree size and landscape position

Tree growth may be limited by microsite conditions, including slope position. In this study tree dimensions did not differ greatly between trees found at different slope positions (Table 4.6). *Eucalyptus camaldulensis* trees tended to support a greater canopy volume for their size at lower slope positions and least for the upper slope position. *Eucalyptus leucoxylon* trees had larger height:canopy volume ratio for trees in mid and upper slope positions, compared to lower slope positions. However, in both species, trees found on upper slopes had a lower proportion of intact canopy than at other slope positions. *Eucalyptus camaldulensis* trees seemed to be particularly affected by slope position, with trees on the upper slope only having an average of 53% intact canopy.

4.3.4 Tree condition

Overall, *E. camaldulensis* and *E. leucoxylon* paddock trees appeared to have good canopy condition. Trees had approximately 80% canopy intact, with a projected foliage cover of about 50% and low levels of epicormic growth (Table 4.7). However, individual tree condition varied to some degree. Four out of 30 *E. leucoxylon* trees had a canopy extent of less than 60% as did three out of 30 *E. camaldulensis* trees. Six of these seven trees in poor condition had projected foliage cover of less than 50% and all trees had moderate to high levels of epicormic growth (10-40% of canopy). Two *E. leucoxylon* trees had epicormic growth of over 20%, while there were 5 *E. camaldulensis* trees with very high levels of epicormic growth (40-50%).

The majority of trees exhibiting poor canopy condition were located <70m to the nearest neighbour but two trees were quite isolated (140m and 170m to nearest neighbour). Trees are potentially more susceptible to the effects of insect defoliation, soil compaction due to stock camping or exposure to wind and drying conditions as tree isolation increases. However, the degree of isolation (distance to nearest neighbour) was not significantly correlated with percent canopy extent ($r^2=0.04$), percentage epicormic growth ($r^2=0.0$) or foliage density ($r^2=0.0$) in either species.

Table 4.6: Dimensions of *E. camaldulensis* and *E. leucoxylon* paddock trees at different slope positions (mean ± S.E.).

Number of individuals at each slope position in parentheses. *DBH* = Diameter at breast height. Intact canopy volume calculated by multiplying canopy volume by canopy extent.

Measurement	*E. camaldulensis*			*E. leucoxylon*		
	Lower (8)	Mid (17)	Upper (5)	Lower (3)	Mid (18)	Upper (9)
DBH (cm)	111 ± 23	155 ± 16	174 ± 12	83 ± 19	98 ± 8	94 ± 15
Height (m)	16 ± 2	18 ± 1	18 ± 1	19 ± 3	19 ± 1	19 ± 1
Canopy volume (m^3)	4210 ± 1266	3943 ± 445	3571 ± 734	2304 ± 1090	2786 ± 325	2879 ± 477
Intact canopy volume (m^3)	3645 ± 1125	3194 ± 383	1895 ± 288	1875 ± 879	2346 ± 268	2077 ± 470
Ratio height: canopy volume	264	220	203	123	149	155
Ratio height: intact canopy volume	228	178	108	100	125	112

Table 4.7: Measures of tree canopy condition (mean ± S.E.) of *E. camaldulensis* and *E. leucoxylon* trees (n=30 each species).

Species	Canopy extent (%)	Foliage density (%)	Epicormic growth (%)
E. camaldulensis	79.8 ± 3.0	47.2 ± 2.2	12.0 ± 2.6
E. leucoxylon	82.7 ± 2.8	48.3 ± 2.5	7.5 ± 1.2

Table 4.8: Number of trees with different levels of tree use by livestock

Species	Low	Medium	High
E. camaldulensis	17	10	3
E. leucoxylon	16	11	3

4.3.5   Tree use by livestock

Patterns of tree use by livestock were similar in both species, with the majority of trees experiencing low tree use by stock. In both species, three trees had very high stock use indicated by high levels of soil compaction, little ground cover and high levels of stock faeces. There did not appear to be any trend with the level of tree use and tree variables such as canopy volume, canopy area or distance to nearest neighbour (all correlations were non-significant, data not shown).

4.3.6   Genetic diversity measures

A total of 81 alleles were detected amongst the eight microsatellites in the population of thirty *E. leucoxylon* scattered trees surveyed, an average of 10.1 alleles per locus (Table 4.9). In the sample of thirty *E. camaldulensis* individuals, 46 alleles were detected at seven microsatellite loci, an average of 6.6 alleles per locus. *Eucalyptus leucoxylon* paddock trees contained a higher proportion of rare alleles (<5% frequency) than *E. camaldulensis* trees (48% c.f. 33%, respectively). In both species observed heterozygosity was high, and observed heterozygosity was greater than expected heterozygosity. Wright's inbreeding coefficient (*f*) measures the excess homozygosity caused by selfing (i.e. the fraction of individuals with alleles identical by descent, due to inbreeding or selfing); a positive value indicates inbreeding. In both species, no inbreeding was detected in the adult populations. Indeed, the negative value of *f* indicates a significant excess of heterozygotes (relative to expectations under a model of random mating) amongst the surveyed paddock trees. In addition, all multi-locus

genotypes of adults of each species were unique and the mean relatedness of adults of both species was close to zero, indicating that all adults sampled were unrelated.

Although spatial genetic structure has been shown to occur over small distances in eucalypt populations (e.g. up to 25m in *E. globulus* (Skabo *et al.* 1998)), sample sizes were too small to make comparisons of such fine-scale genetic structure in either species. Genetic structure was assessed over a distance of 250m which is approximately the maximum distance individual trees are isolated from their nearest neighbours at each site. *Eucalyptus camaldulensis* trees showed weak but significant spatial structuring of genotypes to 100m (*P*=0.002) and in the 151-200m class (*P*=0.009), but no structuring at distances of 201-250m (Figure 4.5a). Large scale genetic structure was assessed over the maximum distance over which trees were surveyed. On a landscape scale, *E. camaldulensis* had significant structuring of genotypes to ~500m (*P*=0.005) but the correlation of genotypes was close to zero in more distant classes (Figure 4.5c). In *E. leucoxylon* significant fine-scale genetic structure was detected to a distance of 50m (*P*=0.023), which declined to zero in further distance classes (Figure 4.5b). On a landscape scale the correlation of *E. leucoxylon* genotypes was close to zero for all distance classes (Figure 4.5d).

Table 4.9: Genetic diversity measures for *E. camaldulensis* and *E. leucoxylon* paddock trees (n=30).

Eucalyptus camaldulensis trees were genotyped at 7 microsatellite loci and *E. leucoxylon* trees were genotyped at 8 microsatellite loci.
n = number of individuals; N_a = Mean number of alleles; $N_a \geq 5\%$ = Mean number of alleles with a frequency $\geq 5\%$; H_o = Mean observed heterozygosity; H_e = Mean expected heterozygosity; f = Mean Wright's inbreeding coefficient; R = Mean relatedness (Queller & Goodnight 1989). Values in parentheses are standard errors of the mean.

	n	N_a	N_a ($\geq 5\%$)	H_o	H_e	f	R
E. camaldulensis	30	6.57 (0.9)	4.43 (0.48)	0.813 (0.04)	0.707 (0.04)	-0.153 (0.04)	-0.036 (0.22)
E. leucoxylon	30	10.13 (1.0)	5.25 (0.80)	0.835 (0.02)	0.764 (0.04)	-0.104 (0.04)	-0.039 (0.18)

a) *E. camaldulensis* – fine scale
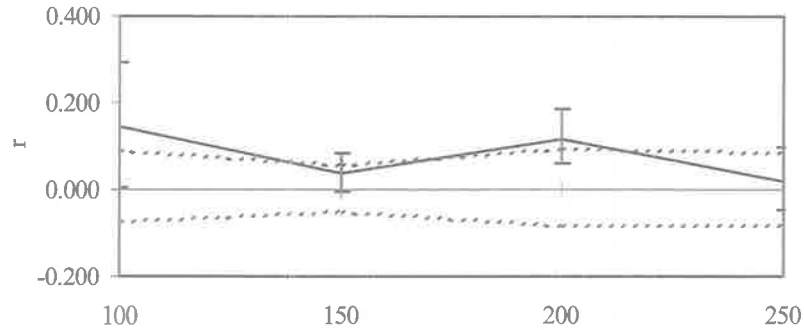
b) *E. leucoxylon* – fine scale
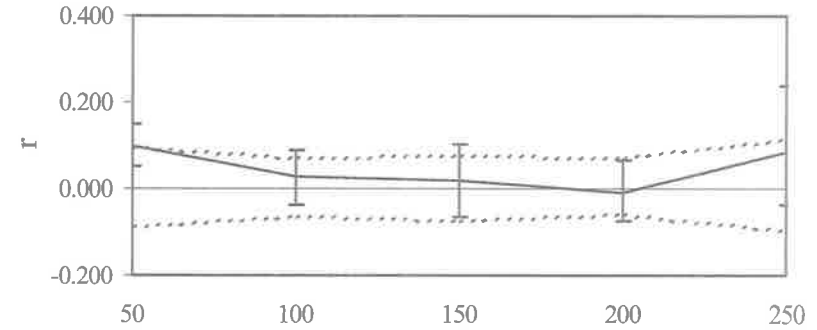
c) *E. camaldulensis* – large-scale
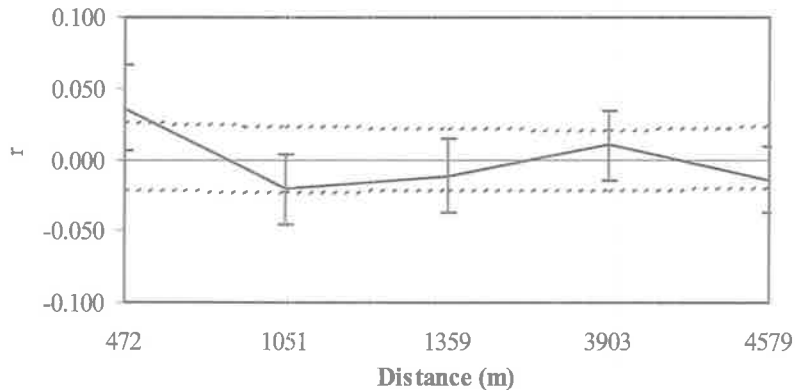
d) *E. leucoxylon* – large-scale
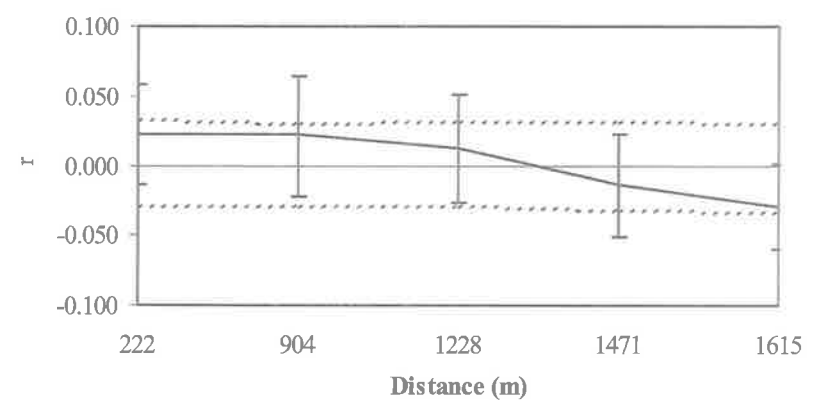
Figure 4.5: Correlograms of fine- and landscape-scale genetic structure of *E. camaldulensis* and *E. leucoxylon* scattered trees.

Upper and lower 95% confidence intervals (determined by bootstrap resampling) about the null hypothesis of no spatial structure are indicated by dotted lines. For the fine-scale analysis there were insufficient replicates of *E. camaldulensis* trees in the 0-50m distance class; these were pooled into the 100m class. For the large-scale analysis five equal distance classes were used for each species determined by the maximum distance individuals were separated by.

4.4 Discussion

In this chapter I explored some of the physical, demographic and genetic characteristics of a sample of scattered trees of two woodland eucalypt species that occur in the Mt Lofty Ranges in South Australia. *Eucalyptus camaldulensis* trees had a relatively continuous distribution across the study area and did not intermix with other eucalypt species. Whether this is representative of the original distribution of trees at the Tungkillo site or whether other eucalypt species have subsequently been cleared is hard to determine. There is some anecdotal evidence that *E. leucoxylon* was once common in the paddocks surveyed and that this species were preferentially cleared because they compete with pasture grasses for moisture and nutrients (M. Collins, pers. comm.). In addition, *E. camaldulensis* paddock trees were found across a range of topographies. In contrast, *E. leucoxylon* trees were primarily located on mid and upper slopes. While the topographical distribution of paddock trees may partially reflect their pre-clearance distribution also, the current pattern of tree distribution may also be the result of patterns of vegetation clearance. Few paddock trees of either species were located on lower slopes as these are the areas that contain the deeper and more fertile soils most suitable for agriculture (Paton *et al.* 1999) and potentially have been preferentially cleared.

While a number of trees of both species had high levels of epicormic growth, the canopy condition of these trees was being maintained, and overall, the majority of trees surveyed appeared to be in good condition. In this survey of a small number of paddock trees of two species, the spatial separation of trees did not appear to directly impact on tree condition, but other factors such as slope position seem to be a clearer determinate of tree condition. This was especially so for *E. camaldulensis*, which naturally is adapted to moist soil conditions; trees found on upper slopes were smaller and supported less canopy than trees on mid or lower slopes. The negative impacts of spatial isolation of trees, for instance, increased wind exposure and increased susceptibility to insect attack (e.g. Lowman & Heatwole (1992) showed paddock trees had twice the rate of insect defoliation as trees in intact woodland) may be balanced by the positive effects. All paddock trees were of large size, and thus can be expected to have a correspondingly large root volume. This means that individual trees are able to intercept large volumes of water and nutrients to maintain growth and have unimpeded

access to sunlight for photosynthesis, which may help buffer against environmental and ecological stresses. High levels of stock use of individual trees did not seem to visibly impact on their condition. Again, there may be positive and negative effects of stock use – soil compaction may lead to a decline in tree health, but the addition of nutrients through stock urine and faeces may enhance growth.

In agricultural systems, tree regeneration is suppressed through grazing by livestock, rabbits or native herbivores, competition from weeds and non-native pasture grasses or altered soil conditions (Yates & Hobbs 1997; Saunders *et al.* 2003; Dorrough & Moxham 2005). In this survey of scattered eucalypt trees located on properties with concomitant grazing, no regeneration of eucalypts was observed. While I did not intentionally exclude small trees from this survey, in reality, there were very few young trees in the landscape. A large proportion of the scattered trees surveyed had a DBH of 100cm or greater (77% of *E. camaldulensis* trees; 47% of *E. leucoxylon* trees). Based on a diameter growth rate of 5mm/yr (estimated from tree ring data for *E. marginata* (Stoneman *et al.* 1997), a tree girth of 100cm is equivalent to 200 years of age, and the youngest trees surveyed were estimated to be 45 and 71 years of age for *E. camaldulensis* and *E. leucoxylon*, respectively. This is consistent with a lack of eucalypt recruitment in other areas of South Australia over the last 50-150 years (Carruthers *et al.* 2004).

However, it should be noted that these age estimates may not be precise since it is problematic applying growth rate data from other eucalypt species to estimate the age structure of *E. camaldulensis* and *E. leucoxylon* trees when growth rate data are unknown for both species. I chose Stoneman *et al.*'s (1997) estimate because it is the largest published data set for any eucalypt species. However, *E. marginata* is a forest tree species and has an upright growth habit, in contrast to the more spreading woodland habit of *E. camaldulensis* and *E. leucoxylon* trees. Differences between woodland species and forest species in the amount of tissue allocated to growth in stem diameter as opposed to stem height may indeed alter age estimations based on tree girth measurements. Secondly, *E. marginata* trees were surveyed in continuous forest where growth rates may be limited by competition with other individuals or other species. Surveys of paddock tree size distribution have shown that *E. camaldulensis* and *E. leucoxylon* paddock trees tend to have larger trunk and canopy diameters than trees in

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high cover sites (this chapter, Carruthers *et al.* 2004), and in this survey I found that tree size of both species was strongly correlated with distance to nearest neighbour for trees within 50m of another individual, indicating a degree of density dependent growth. Thus, it is likely that annual growth rates of paddock trees may be greater than trees in continuous forest due to reduced competition for space and resources, and may differ between individuals dependent on the length of time since the removal of surrounding vegetation, making it difficult to accurately estimate age from tree size data for these trees. Clearly though, the skewed age structure of remnant paddock trees and lack of recruitment is of grave concern. Long-term studies of other woodland eucalypt species have shown an alarming rate of decline for mature trees affected by habitat clearance and fragmentation (Ozolins *et al.* 2001; Saunders *et al.* 2003), estimated to be 0.54-2.5% per year (Reid & Landsberg 1999). Thus, it is essential that the issue of eucalypt recruitment to agricultural environments be addressed over the coming years, before seed supply and/or seed quality declines beyond a critical point as a result of the loss of mature reproductive trees.

Overall, the average density of conspecifics was 0.86 trees/ha for *E. leucoxylon* trees and 1 tree/ha for *E. camaldulensis*. These estimates are consistent with paddock tree densities for other regions of Australia. Martin *et al.* (2004) reported similar densities of *E. albens* in cleared paddocks (1 tree/ha) in north western NSW, while Ozolins *et al.* (2001) reported an average tree density of 0.3 tree/ha for scattered eucalypt trees (all species) in central NSW and Carruthers *et al.* (2004) found paddock tree densities (all species) varied from 0.25-3 trees/ha in south-eastern South Australia. The average distance between conspecific scattered trees at Tungkillo and Flaxley was 65-70m, but varied greatly across the two sites. The average isolation distance of these trees was very similar to that reported in Ozolins *et al.* (2001) (54m in pasture) and Gibbons & Boak (2002) (80m).

Through historical surveys, Ozolins *et al.* (2001) have shown that the degree of tree isolation has increased in 30 years from 63 to 72m. Indeed, the loss of mature paddock trees is predicted to have dramatic effects on the landscape in which they occur. For example, in a survey of agricultural properties in Victoria, Dorrough & Moxham (2005) predicted that the loss of paddock trees would reduce tree cover on agricultural properties by 30% and Gibbons and Boak (2002) estimate that with the loss of paddock

trees from their study sites, the distance to tree cover would increase from 80 to 144m. The impacts of increasing tree isolation on the flora and fauna that depend on scattered eucalypt trees are unknown because, at this stage, we know very little of the ecological dynamics of such a system. If we are concerned with preserving the ecological and evolutionary potential of these trees, it is important that we gain an understanding of some of the fundamental processes occurring in this system.

As described, *E. leucoxylon* scattered trees had a patchier distribution than *E. camaldulensis* trees that most likely reflects its pre-habitat clearance distribution. The patchiness of *E. leucoxylon* trees means that on a much broader landscape scale, *E. leucoxylon* trees occur at a very low density. In addition, *E. leucoxylon* scattered trees occur in conjunction with a number of other eucalypt species, in contrast to the *E. camaldulensis* field site which was essentially dominated by a single species. This contrasting distribution of eucalypt species may have different consequences for each tree species, and animal species that depend on them (e.g. pollinators), post-habitat clearance. For example, *E. leucoxylon* is pollinated predominantly by birds (although insect visitors are also common) (Paton & Ford 1977; Ellis & Sedgley 1993). Hopper & Moran (1981) have suggested that a patchy distribution has favoured the evolution of bird pollination in some eucalypt populations as a mechanism for maximising outcrossing and promoting genetic diversity. Does a reduction in tree density have less impact on the mating system of a species utilising a highly mobile pollinator (birds) as opposed to a species reliant on invertebrate pollination? Alternatively, does a continuous distribution of a single species (as that of *E. camaldulensis*) allow pollination systems to be maintained, in contrast to a population that has increased in patchiness (*E. leucoxylon*) following habitat disturbance (that may be further exacerbated by patchiness in flowering also).

The reproductive attributes of scattered *E. camaldulensis* and *E. leucoxylon* trees are to be examined in future chapters but a number of interesting questions can be raised. For example, both species surveyed occur as large trees when physically isolated. Do these large trees that support large canopies also produce large floral crops? If so, how does this impact on their attractiveness to pollinators? Does tree condition impact on floral display? Large floral displays may be attractive to pollinators but do the physical distances that separate trees and the drastic reduction in tree density lead to changes in

pollinator foraging behaviour? For example, the cost of moving between trees may lead to pollinators spending more time foraging on individual trees, altering the mating system of individual trees by increasing the level of geitonogamous selfing. Are there any differences between the responses of insect pollinators to reduced tree densities, as opposed to the more mobile bird pollinators? While I have discussed the impact of tree distribution on a particular guild of animal species (pollinators), the implications for other vertebrate and invertebrate species dependent on eucalypt species can be considered similarly.

I found that the standing genetic diversity of scattered trees in both *E. camaldulensis* and *E. leucoxylon* populations was high, both in terms of the number of alleles present and the levels of heterozygosity. I detected more alleles per locus, more rare alleles and a higher level of heterozygosity amongst *E. leucoxylon* scattered trees than amongst *E. camaldulensis* trees, which may be suggestive of a higher natural level of genetic diversity for this species. However, this interpretation may not be correct for two reasons. Firstly, although I sampled multiple selectively-neutral markers, I did not sample the same microsatellite loci in each species and my results may have been unknowingly biased by the choice of loci. Secondly, the source of markers was different for each species: *Eucalyptus leucoxylon* markers were developed from that species, whereas I used *E. globulus* markers for *E. camaldulensis*. Several studies have shown that the level of polymorphism of microsatellite markers declines when transferred to species of increasing taxonomic distance from the source species (Primmer *et al.* 1996; Peakall *et al.* 1998). Thus, it is unclear whether the lower average number of alleles per locus and observed heterozygosity found in *E. camaldulensis* is indicative of lower genetic diversity in this species or whether this is simply an artefact of the markers used.

Nonetheless, values of the number of alleles (N_a) and observed heterozygosity (H_o) for both species are consistent with those reported for other natural populations of eucalypt species using microsatellite markers (Table 4.10). Interestingly, paddock tree populations displayed a significant deficit of homozygotes in comparison to natural populations of other eucalypt species (Table 4.10), which may indicate strong selection against homozygous individuals in the highly modified agricultural environment. While it is encouraging that I detected similar levels of neutral genetic diversity in the paddock

trees as to that found in other natural eucalypt populations, it is unknown whether there is an equivalent diversity of functionally or ecologically significant genetic traits represented in these populations. With the availability of genetic linkage maps for quantitative trait loci of a number of eucalypt species (Marques *et al.* 1998; Brondani *et al.* 2002; Thamarus *et al.* 2002) and the future of genomic research in *Eucalyptus* (Poke *et al.* 2005), this would certainly be an interesting area for future ecological research.

Table 4.10: Comparison of genetic diversity estimates for *E. camaldulensis* and *E. leucoxylon* paddock trees with estimates from natural populations of a range of other *Eucalyptus* species based on microsatellite markers

Species	No. loci	N_a	H_e	H_o	Reference
E. camaldulensis(n=30)	7	6.6	0.71	0.81	This study
E. leucoxylon (n=30)	8	10.1	0.76	0.84	This study
E. globulus (n=168)	8	14.5	0.84	0.85	Jones *et al.* 2007
E. morisbyi (n= 57)	6	10.5	0.77	0.64	Jones *et al.* 2005
E. benthamii (n=72)	22	6.7	0.70	0.66	Butcher *et al.* 2005
E. considennia (n=394)	11	11.2	0.61	0.60	Glaubitz *et al.* 2003
E. sieberi (n=100)	8	18.9	0.87	0.84	Glaubitz *et al.* 2001
E. nitens (n=20)	4	9.5	0.83	0.58	Byrne *et al.* 1996

In plant populations with limited gene dispersal, via either or both, pollen or seed dispersal, spatial structuring of plant genotypes is expected to occur (Peakall & Beattie 1996; Degen *et al.* 2001). Both *E. camaldulensis* and *E. leucoxylon* are known to have a mixed mating system with a high level of outcrossing but also significant selfing (*E. camaldulensis*, t_m=0.75, Moncur *et al.* 1995; *E. leucoxylon* t_m=0.83, Ellis & Sedgley 1993). In addition, significant levels of biparental inbreeding (mating with near neighbours) have been detected in a range of eucalypts (Sampson *et al.* 1995; Butcher *et al.* 2005). Many eucalypts have no obvious adaptations for seed dispersal and seed fall tends to be primarily by gravity, usually to within a distance of two to three heights of the adult tree (Cremer 1977; Pudney 1998). A number of authors have hypothesised that such spatial structuring of genotypes can be expected in eucalypt populations (Griffin 1980; Eldridge *et al.* 1993; Hardner *et al.* 1998). In a test of this hypothesis, Skabo *et al.* (1998) detected significant spatial structuring of RAPD genotypes to a distance of 25m in a natural population of *E. globulus* in Tasmania, while genetic similarity declined greatly at distances greater than 25m up to 14km. I detected weak, but significant spatial genetic structure in both *E. camaldulensis* (up to 100m) and *E. leucoxylon* (up to 50m).

If fine-scale genetic structuring was present in *E. camaldulensis* and *E. leucoxylon* populations prior to vegetation clearance, a number of lines of evidence suggest that tree clearance has had the effect of removing related individuals such that very little spatial structuring can now be detected. Genetic measures indicated a lack of homozygotes in both species, which is in contrast to expectations based upon knowledge of eucalypt mating systems (i.e. the ability of individuals to self) and the limited distance of seed dispersal (which could lead to consanguineous matings). An estimate of adult relatedness indicated that on average all individuals were unrelated (r=-0.03). The loss of related individuals from the population means that all remaining genotypes are more or less unique and consequently that there is very little redundancy in the system. Therefore, the continual loss and removal of scattered trees from the landscape will lead to the erosion of neutral and quantitative genetic diversity both on a local scale and at the landscape scale. Indeed, this may be particularly relevant for trees that are found in remnant patches, where individuals are likely to be closely related and susceptible to the effects of inbreeding and genetic drift as a consequence of habitat fragmentation (Young *et al.* 1996). Paddock trees associated with remnant patches may be a source of additional genetic variation, and as long as gene flow via pollen dispersal is maintained, may help reduce these impacts.

4.5 Conclusions

Paddock trees provide a range of ecosystem services, contributing to land, water and biodiversity conservation as "keystone" structures, and are thus an important resource to be managed. As has been found elsewhere, the surveyed populations of *E. camaldulensis* and *E. leucoxylon* paddock trees in the MLR comprised mostly older-aged individuals and it appears that recruitment has been absent from the paddock tree environment for upwards of 40 years. Consequently, the ageing nature and spatial isolation of trees are priority areas for attention since, as this study shows, paddock trees represent an additional and irreplaceable store of genetic variation that may be important for contributing to species conservation on a landscape scale.

Chapter 5   Flower and seed production in *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees

5.1   Introduction

The consequences of habitat fragmentation (small patch size, reduced population density, increased isolation) have been hypothesised to lead to a reduction in plant fecundity (Severns 2003; Waites & Agren 2004), acting through changes to pollinator characteristics, mate availability, seed dispersal, key disturbance regimes and the operation of edge effects (e.g. invasive species) (reviewed in Hobbs & Yates 2003). Because most angiosperms require biotic vectors to reproduce sexually, the ecological response of pollinator populations (e.g. species composition, pollinator abundance, pollinator behaviour) to habitat fragmentation will play a major role in determining the consequences for plant reproduction. In addition, the extent to which changes in pollinator populations will impact on plant reproduction is also dependent on the plant's breeding system (e.g. self-compatible vs. self-incompatible) and the degree of pollination specialisation (e.g. generalist vs. specialist).

Pollinators are expected to respond to changes in pollen and nectar resource availability brought about by altered plant population size (the number of individuals in a population or patch) and/or population density (the number of individuals per unit area). Optimal foraging theory predicts that visitation rates by pollinators should be higher in dense patches, and that isolated plants should receive fewer visits but more flowers would be probed per plant visit (i.e. the marginal value theorem, Charnov 1976; Pyke 1979; Goulson 2000). In addition, the amount of time pollinators may spend foraging in a patch is a function of the "payoff" for staying in the patch measured against the cost of travelling between patches (Charnov 1976; Goulson 2000). Thus, it is generally predicted that small, isolated patches and/or reduced plant density, the typical consequences of habitat fragmentation, will lead to lower pollinator visitation rates and increased bout lengths. This may have detrimental consequences for plant mating systems through increased geitonogamous pollination (de Jong *et al.* 1992) or mating with relatives (Lamont *et al.* 1993; Hardner *et al.* 1998; Young *et al.* 2000), leading to inbreeding depression in self-compatible species (Charlesworth & Charlesworth 1987), or pollen discounting (Ritland 1991) and reduced mate availability (Young *et al.* 1996;

Vekemans *et al.* 1998) for self-incompatible species. The extent to which this occurs though will be dependent on both the life-history characteristics of the plant species and the pollinator species in question, and the interactions between them.

Indeed, a number of habitat fragmentation studies have shown that plants in fragments receive fewer pollinator visits and flowers receive less pollen, leading to reduced fruit set (e.g. Aizen & Feinsinger 1994a; Steffan-Dewenter & Tascharntke 1999; Fuchs *et al.* 2003; Quesada *et al.* 2003; Waites & Agren 2004). This is often a function of population size, and consequently floral display, with small fragments being the most severely impacted upon (Goodell *et al.* 1997; Donaldson *et al.* 2002; Tomimatsu & Ohara 2002; Severns 2003; Waites & Agren 2004). However, the reverse has also been found to be true for a small number of species, with increased pollinator visits and increased fruit set in small patches (Donaldson *et al.* 2002; Smith-Ramirez & Armesto 2003) or on fragment edges (Montgomery *et al.* 2003). Similarly, some studies have shown that the degree of patch isolation influences plant fruit set, independent of patch size (Groom 1998; Duncan *et al.* 2004), while others have shown no effect (Kolb 2005; Ward & Johnson 2005). However in many systems patch size and patch isolation are highly correlated and it is difficult to assess the effects of each independently. Changes in pollinator composition may be detrimental when co-adapted pollinators are replaced by less efficient pollinators (Vaughton 1996; Gross & Mackay 1998) but, in some cases, introduced pollinators have helped to ameliorate the effects of habitat fragmentation and patch isolation (Aizen & Feinsinger 1994b; Dick 2001; Gross 2001). Thus, it is not always clear how individual species will respond to the changes in population configuration produced by habitat clearance. When multiple species have been examined in the one ecosystem (e.g. Aizen & Feinsinger 1994a; Steffan-Dewenter & Tascharntke 1999; Cunningham 2000; Donaldson *et al.* 2002; Quesada *et al.* 2004), species responses have been idiosyncratic – a number exhibit reduced fruit set, others show no effect, while a small proportion of species have shown an increase in fruit set.

The response of plant species to the extreme form of habitat fragmentation that is being examined in the present study, where all intervening vegetation has been cleared and only low numbers of remnant trees are left ("isolated trees" or "paddock trees"), has been very much less studied. Recently several studies have described the reproductive ecology of remnant pasture trees in a tropical habitat. In the self-incompatible tree

Ceiba pentandra, two isolated trees set no fruits despite massive flowering, whereas two others set large quantities of seed (Gribel *et al.* 1999). Dick (2001) found that isolated trees of *Dizinia excelsa* had three times the level of fruit set than trees found in continuous forest. This was attributed to the presence of introduced Africanized honeybees in pasture habitat, which were absent from continuous forest. In a comparison of isolated remnant trees of *Pachira quinata* with trees in continuous vegetation, Fuchs *et al.* (2003) found that isolated trees had significantly higher flower production than trees in continuous vegetation but only 3% of flowers set fruits as opposed to 6% in continuous vegetation. In Australia, seed production of isolated trees has been examined in two woodland eucalypt species, *E. albens* (Burrows 1995) and *E. melliodora* (Burrows 2000). In *E. albens*, seed production was significantly lower in isolated trees than in trees in woodland environments (Burrows 1995). Also, in *E. melliodora*, seed production, seed viability and germination was greatly reduced in isolated trees as compared to trees in intact woodlands (Burrows 2000). Again, it appears that individual species responses are idiosyncratic.

5.1.1   Reproductive ecology of *Eucalyptus*

Eucalypts are generally considered to have a high reproductive capacity with frequent production of large numbers of flowers and fruits, many ovules and high seed numbers per individual (House 1997). In most eucalypts the flowers are bisexual, and most commonly are held in inflorescences comprised of groups of 3-13 flowers borne on a common stalk. The stamens are the most conspicuous part of the flower; they are often colored and form the attraction unit for pollination (Boland *et al.* 1980). Nectaries are located at the base of the style, which produce a copious flow of nectar (Pryor 1976), an important floral reward for pollinators. Flowers are protandrous, with male and female phases of the flower separated by several days (Hodgson 1976b; Griffin & Hand 1979). However, the development of flowers within and between inflorescences is sequential and gradual so that flowers in male or female phase may be in close proximity, allowing geitonogamous self-pollination to occur (House 1997). Following fertilisation, a woody fruit is developed containing both seed and a mass of smaller unfertilised ovules called "chaff" (Boland *et al.* 1980). The number of viable, fully developed seed in any individual fruit is often quite small under natural conditions but can vary quite considerably between species (e.g. *E. salmonophloia,* two seeds/fruit (Yates *et al.*

1994); *E. albens*, two seeds/fruit (Burrows 1995); *E. camaldulensis*, up to 50 seed/fruit (James & Kennington 1993), *E. caesia*, 150 seed/fruit (Gill *et al.* 1992)). Eucalypt seed is generally very small and the mass of seed within individual fruits compared with chaff is often as little as 5% (Pryor 1976). However, many studies suggest that eucalypt species have very high germination rates (e.g. *E. crebra* 77%, *E.melanophloia* 100% (Burrows & Burrows 1992); *E. salmonophloia* 67-100% (Yates *et al.* 1994); *E. melliodora* 91% (Burrows 2000)). Seed dissemination occurs when the woody capsule dries out and dies, and is largely under the influences of gravity and agitation by the wind, generally dispersing to a maximum distance of up to two heights of the tree (Cremer 1966; Pudney 1998).

5.1.2   Breeding system and inbreeding depression in *Eucalyptus*

The majority of eucalypts studied to date have been shown to be self-compatible, and, as such, are considered to have a mixed mating system with preferential outcrossing (reviewed in Moran & Bell 1983). The term "preferential" outcrossing has been employed since experiments have shown that with the application of equal measures of self and cross pollen to flowers, proportionally more outcrossed than selfed seeds are produced (Hodgson 1976a; Griffin *et al.* 1987). Pre-zygotic self-incompatibility mechanisms have been demonstrated in only a few eucalypt species (*E. woodwardii*, Sedgley & Smith 1989; *E. spathulata*, *E. platypus* (Sedgley & Granger 1996). In these experiments, pollen-tube penetration and fertilisation were reduced following self-pollination compared with cross-pollination. It appears though that post–zygotic incompatibility mechanisms are more common in eucalypts (Potts & Wiltshire 1997; Pound *et al.* 2003a).

A reduction in capsule production, seed yield and seedling vigour have been shown to occur after self pollination compared with cross-pollination for many species (reviewed in Hardner & Potts 1995b). The reduction in seed yield due to selfing can be great in many species (for example, 84% in *E. globulus* (Hardner *et al.* 1998), 95% in *E. woodwardii* (Sedgley & Griffin 1989)), but less severe in others (e.g. 7% in *E. regnans* (Eldridge 1970), 11% in *E. urophylla* (Eldridge 1978)). Post-zygotic self-incompatibility is frequently detected during the embryo developmental stage, when zygotes fail to divide or to develop normally (Sedgley & Granger 1996; Pound *et al.*

2003b). The failure to develop appears to be dependent on embryo genotype, for example, seed abortion may occur due to homozygosity for deleterious recessive genes (Griffin *et al.* 1987) or due to maternal resource allocation (James & Kennington 1993). Inbreeding depression due to selfing may not always be apparent at earlier seedling stages (for example, Hardner & Potts (1995a) found no effect of pollination type on germination rate or survival to 43 months in *E. globulus)* but does occur over the lifetime of the plant. Hardner *et al.* (1998) reported significantly slower growth of selfed *E. globulus* compared to open- and cross-pollinated individuals over a four year period. Similarly, in a long-term field trial of *E. regnans,* Hardner & Potts (1997) found that the survival of self-progenies was 18% at 15 years compared to 55% for outcrossed progenies.

5.1.3   Floral and seed production in isolated *Eucalyptus* trees

As for the majority of other eucalypts, *E. camaldulensis* and *E. leucoxylon* are known to be self-compatible and are therefore potentially susceptible to inbreeding depression when mating conditions are altered through habitat clearance. Several studies to date have suggested that eucalypt paddock trees have poor reproductive performance in comparison to trees found in intact woodland habitat (Burrows 1995; Burrows 2000; Butcher *et al.* 2005). In this chapter I explore a number of aspects of the reproductive ecology of *E. camaldulensis* and *E. leucoxylon* paddock trees, firstly to determine the reproductive status of these trees, and secondly, to investigate what factors (if any) may account for variation in reproductive output of these trees.

Firstly, I performed a rapid survey of flower and fruit production in *E. camaldulensis* and *E. leucoxylon* paddock tree populations to determine what proportion of the population were reproductive and the relative reproductive effort of these trees. I then measured several aspects of flower and fruit production for individual trees. Theory predicts that isolated plants should receive fewer pollinator visits but that pollinators should increase their bout lengths on isolated plants. Potentially this would lead to pollination limitation and increased geitonogamous selfing, resulting in lower fruit production in increasingly spatially isolated trees. However, experiments have shown that pollinators respond to floral display size, such that plants with a greater floral display receive more visits by pollinators than smaller individuals with few flowers, but

pollinators visit a smaller proportion of flowers on plants with a large floral display (Paton & Ford 1982; Geber 1985; Klinkhamer *et al.* 1989; Klinkhamer & de Jong 1990). Thus this suggests that large plants may maintain higher outcrossing rates and therefore have higher fruit production. In Chapter 4 I found that the majority of isolated trees are large in size, both in terms of DBH and canopy volume. To investigate these two contrasting outcomes I correlated measures of tree size and tree spatial isolation with seed and capsule production and germination rates.

On a population scale, I tested whether fruit and seed production differed between plants found in natural vegetation and paddock trees, as it is likely that pollinator behaviour and/or number of pollinators differs between the two vegetation types. For paddock trees I tested whether local conspecific tree density (low vs. medium vs. high) impacts on seed and capsule production as pollinators may respond to plant distribution on a scale larger than the individual tree. In Chapter 4 I found that trees on upper slopes supported smaller canopies for their size and appeared to be stressed. In this chapter I tested whether aspects of flower and fruit production differed for trees at different slope positions as these trees are likely exposed to differing environmental conditions. Finally, I tested whether seed and capsule production differed between different parts of the canopy, since bird pollinators often enter the upper canopy on a foraging bout and this can lead to variation in outcrossing rate across the tree canopy (Hingston & Potts 2005).

Overarching these results is the comparison of a species with predominantly insect-mediated pollination, *E. camaldulensis*, and predominantly bird-mediated pollination, *E. leucoxylon*. Due to the differences in physical size and mobility of these pollinators insect-pollinated *E. camaldulensis* paddock trees are more likely to suffer restricted pollination services than *E. leucoxylon*, as insects are less likely to overcome the spatial isolation and increased dispersion of trees in the landscape than birds.

5.2 Methods

5.2.1 Study sites

Eucalyptus camaldulensis paddock trees were located on agricultural properties near Tungkillo and *E. leucoxylon* paddock trees were located on properties near Flaxley, South Australia, as described in Chapter 4. Trees were also sampled in intact remnant vegetation as described in Chapter 4.

5.2.2 Flowering characteristics of *E. camaldulensis*

Eucalyptus camaldulensis inflorescences are axillary and are held on angular peduncles up to 2.5cm long. The small buds have a beaked operculum and occur in umbels of five to thirteen (typically seven) buds. Flowers are cream-coloured and flowering occurs in late November to February (Paton *et al.* 2004a), though flowering is usually relatively synchronous (i.e. individuals overlap in peak flowering) in any one area and lasts 3-4 weeks during this time (K. Ottewell, pers. obs.). Observations of floral visitors to *E. camaldulensis* paddock trees indicate a suite of generalist pollinators including native bee species, introduced honeybees (*Apis mellifera*), flies, ants and wasps (K. Ottewell, pers. obs.). No birds were observed feeding on nectar or pollen of paddock trees at Tungkillo (K. Ottewell, pers. obs.), though birds have been observed on *E. camaldulensis* trees in other regions (D. Paton, pers. comm.). The fruits of *E. camaldulensis* are pedicellate and hemispherical, 0.6 x 1cm in size (Brooker & Kleinig 2001). Seed shed occurs when drying weather conditions cause the valves to open and capsules are shed from the tree. Again, seed shed is more or less synchronous in the population, occurring in August/September at Tungkillo, when the prevailing winds turn northerly (Pudney 1998, K. Ottewell, pers. obs.). Typically, all capsules are shed from the tree at this time but occasionally, following heavy flowering and fruit production, a proportion of capsules may be held in the canopy until the following year (D. Paton, pers. comm.). *Eucalyptus camaldulensis* is known to be a species that reliably produces large seed crops at regular intervals (Boland *et al.* 1980), however, depending on environmental conditions, *E. camaldulensis* may fail to flower in some seasons after an especially heavy flowering season (typically, once every 3-4 years (D. Paton, K. Ottewell pers. obs), and in the following year only a light seed crop, if any, is produced.

5.2.3   Flowering characteristics of *E. leucoxylon*

Eucalyptus leucoxylon inflorescences are axillary and simple, the peduncles are slender and up to 1.1cm long. Buds have a beaked operculum and occur in umbels of two, or more commonly, three buds. Flower colour in *E. leucoxylon* ranges from cream through to pink or red and flowering occurs predominantly in May through to January at Flaxley. There is some asynchronicity in flowering in *E. leucoxylon*, with individuals reaching peak flowering at different times and spot flowering occurring throughout this period (K. Ottewell, pers obs). Fruit are held on long pedicels and are barrel-shaped, up to 1.2 x 1.2cm in size. Valves are 4-6 and occur below the rim level (Brooker & Kleinig 2001). Seed shed occurs over a much longer time period in *E. leucoxylon* than in *E. camaldulensis*, from February through May, and depending on environmental conditions some capsules may be held in the canopy for more than one flowering season. Current season capsule cohorts are readily distinguishable from previous season cohorts. Current season capsules retain some colour and have a smooth outer surface, while previous season capsules are dull in colour and have a roughened, sometimes cracked surface. Current season capsules were always collected to ensure results were applicable to flowering and other measurements taken during that season.

5.2.4   Surveys of reproductive effort of paddock trees

Both populations of paddock trees were surveyed for relative reproductive effort pre- and post-flowering over two years. Relative reproductive effort was measured on a semi-quantitative scale: 0 = no reproductive units present (buds or capsules); 1 = reproductive units present on 1-35% of branches in canopy; 2 = reproductive units present on 36-65% of branches and 3 = reproductive units present on 66-100% of branches. This essentially measured the number of trees that had no reproductive output, low, moderate or high reproductive output relative to other trees in the population. *Eucalyptus camaldulensis* paddock trees were surveyed in December 1999 (pre-flower) and April 2000 (post-flower) (*n*=42 trees) and in January 2002 (pre-flower) and May 2002 (post-flower) (*n*=41 trees). While similar numbers of trees were surveyed in each year, there was some discrepancy in the particular individuals measured in each year; therefore some individuals were only surveyed in one year and not the other. *Eucalyptus leucoxylon* paddock trees were surveyed in August 2000 (pre-flower) and April 2001 (post-flower) (*n*=32) and July 2002 (pre-flower) and May 2003

(post-flower) (*n*=36). The majority of individuals surveyed in each year were the same though four trees were surveyed in 2003 and not in 2001.

5.2.5   Flowering and fruit set measurements

Floral counts were performed on a subset of *E. camaldulensis* trees (*n*=22) in late December 1999 prior to the trees flowering in early January. Floral counts were also performed on *E. leucoxylon* paddock trees but unfortunately a significant proportion of this data has subsequently been lost. I present data for six trees, surveyed in August 2000 (prior to flowering), though due to low sample size statistical analyses of these data will be limited.

Floral counts were essentially limited to those trees with branches that were readily accessible from the ground. Up to 10 small branches (~0.7-1m in length) were tagged haphazardly on the tree and counts done of the number of leaves, the number of floral units (umbels of buds) and the number of buds per umbel. The counts were performed at two periods: immediately prior to anthesis and at capsule maturity, prior to capsule dehiscence. To standardise comparisons of the level of reproductive output between trees (to eliminate the effect of differences in branch size), the number of floral units per leaf unit was used. The mean ratio of number of buds to leaves, the mean ratio of number of umbels to leaves and the mean number of buds per umbel, were used as measures of intensity of floral display.

Counts of the number of capsules, number of leaves and number of capsules per umbel were conducted on *E. camaldulensis* trees in late April/early May 2000, prior to capsule dehiscence and on *E. leucoxylon* trees in April 2001. Counts were performed on trees in the same manner as for the flower counts and data presented on the ratio of number of capsules to number of leaves, average number of capsules per umbel and ratio of number of umbels of capsules to number of leaves.

5.2.6   Seed production in scattered *E. camaldulensis* and *E. leucoxylon* trees

Mature capsules were collected from *E. camaldulensis* trees at Tungkillo in May-June 2000 (6 months after flowering) and in Sept 2002 (7 months after flowering). *E. camaldulensis* failed to flower in the 2001 season. Mature capsules were collected from

E. leucoxylon trees at Flaxley in April 2001 (7 months after flowering) and May 2003 (7 months after flowering). In both cases, the seed was mature at this stage and had not been released. Seed capsules were collected from paddock trees and from trees found in intact woodland vegetation for both species. Paddock trees varied in their degree of isolation – from <20m to the nearest conspecific to >150m to the nearest conspecific. Statistical tests were performed on seed and capsule data in comparison to patterns of local tree density. Tree density was estimated from aerial photographs and trees were characterised into four density categories: natural (estimated >50 trees in 250m radius), high density (estimated >50 trees in 250m radius but no understorey), medium density (5-15 trees in 250m radius) and low density (0-5 trees in 250m radius).

Twenty to 100 capsules were collected from each tree depending on availability. Capsules were collected from up to 10 different branches from various positions (around the perimeter of the canopy and from inside the canopy) in the tree up to a height of approximately 6m using secateurs mounted on a telescopic aluminium pole. When picking capsules from the branch, only one capsule per umbel was selected and capsules were collected from as many different umbels as possible. Groups of capsules collected from different branches of individual trees (hereafter referred to as seedlots) were kept in separate containers and treated independently (i.e. up to 10 replicate seedlots per tree per flowering season). The capsules were dried for approximately one week in an oven at 32°C until the valves opened and the capsule contents shed. Capsules were vigorously shaken and individually inspected to ensure complete evacuation of capsule contents.

Eucalypt capsules contain seed and chaff in various proportions (the chaff consists of unfertilised and aborted ovules). In *E. camaldulensis* the seed is of a similar colour and size (0.1mm) to the chaff so it was virtually impossible to separate the seed from the chaff by hand or by mechanical means. Therefore, the following results relate to the capsule contents as a whole rather than seed per se. To allow comparisons between species, *E. leucoxylon* capsule contents were also treated in the same manner. The weight of the capsule contents, the dry weight of the empty capsule and the diameter of capsules (measured at the widest point) were determined. In order to compare patterns of seed production between individuals, I used the ratio of weight of capsule contents to the weight of an empty capsule. This controls for differences in capsule size between

trees. A low value indicates trees produced more capsule contents for the size of the capsule.

5.2.7   Seed germination

As mentioned previously, seed was unable to be separated from chaff therefore, as a standard measure, 100mg of the capsule contents (seed + chaff) of each seedlot were used in the germination trials. The capsule contents were spread on a layer of filter paper placed on water-soaked vermiculite in clear plastic takeaway food containers. The seeds were germinated at room temperature (average 22°C) away from direct sunlight. Counts of the number of germinants were performed every two days from 5 days to 21 days. Germination rate was calculated as the maximum number of germinants after 21 days per 100mg of capsule contents and used as a surrogate measure of seed set.

5.2.8   Statistical analyses

All statistical analyses were performed in the statistical software package SPSS v12. All variables were checked for normality of data (Kolmogorov-Smirnov test) and when appropriate homogeneity of variances (Levine's test). Transformations (e.g. log_{10}, square root) were applied to data that failed these tests to ensure assumptions of normality and homogeneity of variances for statistical tests were met. When data transformations failed to normalise data, non-parametric tests were employed.

A number of ANOVA tests were performed on groups with unequal sizes, which violates the assumptions required for ANOVA. In all cases, standard ANOVA F estimates were checked against the Brown-Forsythe estimate of F provided in SPSS, which is more robust to unequal sample sizes. In cases where the two estimates of F did not vary greatly, the standard value of F is reported.

5.2.8.1   *Data reduction*

In this chapter I perform correlations of a number of tree physical size and demographic characteristics (as measured in Chapter 4) with measures of flower and fruit production. Tree physical characteristics were all highly correlated ($P<0.01$), as were distance to nearest neighbour and distance to nearest conspecific ($P<0.01$) (data not shown). Factor

analysis was applied to reduce the number of variables required for correlation analyses. For *E. camaldulensis*, factor analysis using principal components methods revealed that two principal components explained 87% of the variance in the data (Component 1 = 54%, components 1 + 2 = 87%). The first component essentially related to tree size, explaining the majority of variance for component 1 and the least for component 2 (Table 5.1). The second component related to the two demographic variables, distance to nearest neighbour and distance to nearest conspecific, with both variables explaining the greatest amount of variation in component 2 and the least in component 1 (Table 5.1). For *E. leucoxylon*, two principal components explained 87% of the variance in the data (Component 1 = 74%, components 1+2 = 87%). As for *E. camaldulensis*, the first principal component related to tree size and the second to measures of tree isolation (Table 5.1). The extracted component variables were used in all correlation analyses with flower and fruit variables, as the components are representative of the original variables. For both species, principal component 1 is referred to as "*Tree Size*" and principal component 2 is referred to as "*Tree Isolation*".

Table 5.1: Rotated component matrix from factor analysis of tree size and demographic variables of *E. camaldulensis* and *E. leucoxylon* paddock trees.

Principal Components Analysis and Varimax with Kaiser Normalisation rotation. Rotation converged in 3 iterations for both species.

	E. camaldulensis Component		*E. leucoxylon* Component	
	1	**2**	**1**	**2**
DBH	.711	.549	.512	.787
Height	.836	.376	.830	.318
DBH*Height	.729	.518	.657	.717
Canopy Area	.915	.153	.850	.357
Canopy Volume	.956	.115	.893	.424
Intact Canopy Volume	.913	.010	.939	.180
Distance to nearest neighbour	.131	.965	.300	.863
Distance to nearest conspecific	.146	.951	.165	.860

5.3 Results

5.3.1 Reproductive effort

All *E. camaldulensis* and *E. leucoxylon* paddock trees produced buds in the two years surveyed and, with the exception of one *E. camaldulensis* individual and three *E. leucoxylon* individuals, produced capsules in both years also (Table 5.2). For *E. camaldulensis,* patterns of reproductive effort for bud production were remarkably similar between the two years, with the majority of trees (62% and 61% in 2000 and 2002 respectively) being scored as having high levels of bud production. However, patterns of capsule production differed between the two years, with a greater proportion of trees being scored as having low capsule production in 2002 compared to 2000 (37% to 24% respectively). In both years approximately 60% of trees were scored as having the same level of bud production as capsule production, which suggests that these trees received adequate pollination services (that is, the majority of buds were converted to capsules). In 2000, ~40% of trees had high bud production but only moderate capsule production, suggesting that a proportion of trees were pollination limited. In 2002, 12% of trees were scored as having high bud production but only very low capsule production, indicating that pollination services were further limited in this year.

Patterns of reproductive effort differed between the two survey years for *E. leucoxylon* paddock trees. In 2001, close to 80% of trees surveyed had high bud production while in 2003 only 58% of trees recorded high bud production (Table 5.2). Despite high bud production in 2001, only 31% of trees had high capsule production in 2001, with the greatest proportion of trees having only moderate capsule production. In contrast, in 2003 a greater proportion of trees recorded as having high bud production also had high capsule production. Interestingly, as for *E. camaldulensis* trees, 53-58% of *E. leucoxylon* paddock trees were scored as having the same level of bud production as capsule production. However, in 2001 three *E. leucoxylon* paddock trees were scored as having high bud production and failed to produce any capsules at the time of the survey, indicating severe pollen limitation and/or reproductive failure for these trees.

Table 5.2:　Reproductive effort of *E. camaldulensis* and *E. leucoxylon* paddock trees prior to flowering and post-flowering in two survey years.

Values represent the number of individuals scored in each reproductive effort category and, in italics, the proportion of the total population in each category.

	E. camaldulensis			
Reproductive effort	**2000 Preflower**	**2000 Postflower**	**2002 Preflower**	**2002 Postflower**
None	**0**	**0**	**0**	**1**
	0	*0*	*0*	*0.02*
Low	**4**	**10**	**5**	**15**
	0.10	*0.24*	*0.12*	*0.37*
Moderate	**12**	**17**	**11**	**8**
	0.29	*0.40*	*0.27*	*0.20*
High	**26**	**15**	**25**	**17**
	0.62	*0.36*	*0.61*	*0.41*
Total	**42**	**42**	**41**	**41**
Same category [a]		**25**		**26**
		0.60		*0.63*
Decline one category [b]		**17**		**10**
		0.41		*0.24*
Decline two categories [b]		**0**		**5**
		0		*0.12*

	E. leucoxylon			
Reproductive effort	**2001 Preflower**	**2001 Postflower**	**2003 Preflower**	**2003 Postflower**
None	**0**	**3**	**0**	**0**
	0	*0.09*	*0*	*0*
Low	**1**	**4**	**3**	**13**
	0.03	*0.13*	*0.08*	*0.36*
Moderate	**8**	**15**	**12**	**8**
	0.25	*0.47*	*0.33*	*0.22*
High	**23**	**10**	**21**	**15**
	0.72	*0.31*	*0.58*	*0.42*
Total	**32**	**32**	**36**	**36**
Same category [a]		**17**		**21**
		0.53		*0.58*
Decline one category [b]		**11**		**14**
		0.34		*0.39*
Decline two categories [b]		**1**		**1**
		0.03		*0.03*
Decline three categories [b]		**3**		
		0.09		

[a] Number of trees that were scored in the same reproductive effort category for bud and capsule production. [b] Number of trees scored in different reproductive effort categories for bud and capsule production (for example, High bud production and Moderate capsule production).

5.3.2   Flower and fruit production in *E. camaldulensis* paddock trees

Across the population of *E. camaldulensis* trees surveyed prior to flowering, trees supported an average of 10.4 buds per leaf and an average of 2.3 umbels per leaf (Table 5.3a). In *E. camaldulensis* paddock trees, umbel size ranged from 1-13 buds, with an average of 4.4 buds per umbel. While large trees may potentially be able to produce and sustain a large floral crop, there appeared to be no correlation of measures of bud production with tree size variables (Table 5.3a), indicating that large trees do not necessarily produce a proportionally larger number of buds than smaller trees. The degree of tree isolation also was not correlated with bud production.

Eucalyptus camaldulensis paddock trees produced an average of 4.5 capsules per leaf unit representing an average of 43% of buds having been converted to capsules. Again, capsule production was not correlated with measures of tree size (Table 5.3b), but there appeared to be a positive (though non-significant) relationship between capsule production (number of capsules per leaf unit and number of umbels of capsules per leaf unit) and tree isolation. This indicates that isolated trees are able to maintain a greater number of capsules per leaf unit than trees in close proximity to other individuals. While this may be due to a higher level of fruit production for these trees, it may also be the result of reduced leaf production relative to fruit production.

5.3.2.1   *Slope position and floral and fruit production*

In Chapter 4, I found that *E. camaldulensis* trees found at on upper slopes had smaller canopy volumes and an average of only 53% intact canopy, suggesting trees at higher elevations were stressed. Interestingly, trees on upper slopes had both a higher number of buds and capsules per leaf unit and number of umbels per leaf unit than trees at other elevations (Table 5.4). Trees on upper slopes also produced significantly more capsules per leaf than other trees ($P=0.04$). Again, this may also be due to reduced leaf production in upper slope trees as these were the most stressed of the trees surveyed.

Table 5.3: Mean (± s.d.) ratio of floral units to leaf units and average number of buds or capsules per umbel of scattered *E. camaldulensis* trees (*n*=22), and Pearson's correlations (*r*) with tree size and tree isolation.

Data for the ratio of number of buds/capsules to leaves and the ratio of number of umbels to leaves were $\log_{10}$ transformed to satisfy assumptions of normality. Significance (*P*) in parentheses.

a. Bud production

	Ratio number buds:leaves	Average no. buds per umbel	Ratio number umbels:leaves
Mean (± s.d.)	10.4 ± 8.3	4.4 ± 0.7	2.3 ± 1.7
Minimum	3.16	3.42	0.83
Maximum	38.19	6.08	7.42
Tree Size	-0.09 (0.74)	-0.39 (0.12)	-0.01 (0.98)
Tree Isolation	-0.11 (0.66)	0.20 (0.44)	0.18 (0.49)

b. Capsule production

	Ratio number capsules:leaves	Average no. capsules per umbel	Ratio number umbels:leaves
Mean (± s.d.)	4.5 ± 5.0	2.2 ± 0.8	1.8 ± 1.8
Minimum	0.24	0.65	0.11
Maximum	16.84	3.86	5.90
Tree Size	-0.22 (0.52)	-0.01 (0.99)	-0.18 (0.60)
Tree Isolation	0.47 (0.15)	0.13 (0.70)	0.51 (0.11)

Table 5.4: Mean (± s.d) of measures of floral production for *E. camaldulensis* trees found at different slope positions, and results of one-way ANOVA between slope position and floral characteristics.

Significance (*P*) of ANOVA in parentheses. Superscripts represent groups with significantly different means (*P*=0.05) determined by a Tukey's post-hoc test.

Floral Unit	Slope position	Number floral units/leaves	Number floral units per umbel	Number umbels of floral units/leaves
Buds	Low (*n*=7)	5.6 ± 0.5	4.1 ± 0.1	1.4 ± 0.1
	Mid (*n*=5)	10.3 ± 1.9	4.4 ± 0.2	2.3 ± 0.4
	Upper (*n*=3)	21.2 ± 10.0	4.5 ± 0.3	4.3 ± 1.9
	F	1.29 (0.31)	0.28 (0.84)	1.26 (0.32)
Capsules	Low	1.8 ± 1.2 [a]	2.0 ± 0.4	0.9 ± 0.5
	Mid	3.5 ± 2.4 [a]	2.3 ± 1.1	1.5 ± 1.1
	Upper	15.4 ± 2.1 [b]	2.7 ± 0.3	5.6 ± 0.4
	F	4.44 (0.04)	0.63 (0.55)	3.31 (0.08)

5.3.3 Flower and fruit production in *E. leucoxylon* paddock trees

As mentioned in section 5.2.5, sample sizes for *E. leucoxylon* flower and fruit counts were extremely low. Of the trees surveyed, trees produced an average of 0.86 buds per leaf unit and an average of 0.39 umbels of buds per leaf unit (Table 5.5a). In general, *E. leucoxylon* produce 1-3 buds per umbel. The average number of buds per umbel for paddock trees was 2.2. There was very little correlation between measures of bud production and tree size variables. However, there was a significant negative correlation between the average number of buds per umbel and tree isolation (*P*=0.04), suggesting that isolated trees produce fewer buds per umbel.

Capsule production was extremely low in the *E. leucoxylon* trees surveyed, with an average of 0.08 capsules produced per leaf unit (Table 5.5b). This represents an average fruit set of just 9%. Trees maintained an average of 0.07 umbels of capsules per leaf unit with an average of 1.16 capsules per umbel. Again there was very little correlation between measures of capsule production and tree size or tree isolation.

Table 5.5: Mean ratio of floral units to leaf units and average number of floral units per umbel of scattered *E. leucoxylon* trees (*n*=6), and Pearson's correlations (*r*) with tree size and tree isolation.

Significance (*P*) in parentheses.

a. Bud production

	Ratio number buds:leaves	Average no. buds per umbel	Ratio number umbels:leaves
Mean (± s.d.)	0.86 ± 0.45	2.2 ± 0.23	0.39 ± 0.20
Minimum	0.31	1.70	0.14
Maximum	1.37	2.34	0.61
Tree Size	-0.05 (0.93)	0.39 (0.44)	-0.12 (0.82)
Tree Isolation	-0.18 (0.73)	-0.83 (0.04)	-0.12 (0.83)

b. Capsule production

	Ratio number capsules:leaves	Average no. capsules per umbel	Ratio number umbels:leaves
Mean (± s.d.)	0.08 ± 0.09	1.16 ± 0.09	0.07 ± 0.08
Minimum	0.0	1.04	0.0
Maximum	0.24	1.25	0.21
Tree Size	-0.30 (0.57)	0.77 (0.13)	-0.33 (0.53)
Tree Isolation	-0.60 (0.25)	0.59 (0.30)	-0.56 (0.25)

5.3.4 Fruit and seed production in *E. camaldulensis* and *E. leucoxylon* paddock trees

5.3.4.1 Summary

A total of 3760 capsules were collected from 42 *E. camaldulensis* trees in 2000, an average of 90 capsules per tree. In 2002, fewer unopened capsules were available on trees so only 2397 capsules were collected from 35 individuals (average 69 capsules per tree). The total yield of capsule contents in 2000 was 59g (average 1.4g capsule contents sampled per tree) and 37g in 2002 (average 1.1g per tree). Per seedlot measurements of capsule weight, capsule diameter and weight of capsule contents are given in Table 5.6a, and per tree averages are displayed in Figure 5.1. All capsule and seed measurements showed greater variation between trees than within trees and one-way ANOVA showed that this was significant for all capsule measurements (Weight capsule contents: $F_{49,479}$=463.0, P<0.001; Capsule weight: $F_{49,422}$=216.8, P<0.001; Capsule diameter: $F_{49,427}$=1091.0, P<0.001).

Across the population of *E. camaldulensis* trees there was no significant difference in the weight of capsule contents between years ($F_{1,526}$=0.34, P=0.56), indicating that there was greater variation within years than between years. The mean capsule weight was significantly greater in 2002 than in 2000 ($F_{1,469}$=-43.254, P=<0.001) while capsule diameter was smaller in 2002 than in 2000 (square-root transformed data, $F_{1,474}$=80.9, P=<0.001) (Table 5.6a). A non-parametric Mann-Whitney test showed the ratio of capsule contents to capsule weight was significantly greater in 2002 than in 2000 (Z_{423}=-9.85, P=<0.001). Thus, while the same amount of seed was produced each year, capsules were smaller and heavier in 2002.

In 2001, 454 capsules were collected from 23 *E. leucoxylon* paddock trees (average 19.7 capsules per tree), which yielded 141.2g of capsule contents (average 6.1g per tree). In 2003, 1473 capsules were collected from 29 trees (average 50.8 capsules per tree), yielding 417.2g of capsule contents, an average of 14.4 g per tree. Per seedlot means are found in Table 5.6b and per tree averages are displayed in Figure 5.2. Again, individual trees varied significantly in seed and capsule characteristics (weight capsule contents $F_{30,246}$=10.76, P=<0.001; capsule weight $F_{30,197}$=11.08, P<0.001; capsule diameter $F_{30,197}$=10.64, P=0.001). However, there was no significant difference in seed

and capsule measurements between the two years across the population of *E. leucoxylon* trees (weight of capsule contents $F_{1,275}=0.55$, $P=0.46$; weight of empty capsules, $F_{1,226}=0.10$, $P=0.76$; diameter of capsules, square-root transformed data, $F_{1,226}=0.02$, $P=0.88$; ratio of the weight of capsule contents to the weight of the empty capsule, $F_{1,224}=2.56$, $P=0.11$).

Table 5.6: Characteristics of capsules (mean ± s.d.) collected from *E. camaldulensis* and *E. leucoxylon* paddock trees across two seasons.

n = number of trees from which fruits were collected.

a. *E. camaldulensis*

Measurement	All trees 2000 (*n*=42)	All trees 2002 (*n*=35)	Years combined
Weight of an empty capsule (*mg*)	43.8 ± 18.4	56.2 ± 22.7	48.6 ± 21.1
Weight of capsule contents per capsule (*mg*)	16.5 ± 5.1	16.1 ± 6.2	16.3 ± 5.6
Ratio of weight of capsule contents to the weight of the empty capsules	2.7 ± 1.0	3.9 ± 1.3	3.2 ± 1.3
Capsule diameter (*mm*)	5.3 ± 1.0	4.5 ± 0.8	4.9 ± 1.0

b. *E. leucoxylon*

Measurement	All trees 2001 (*n*=23)	All trees 2003 (*n*=29)	Years combined
Weight of an empty capsule (*mg*)	256.3 ± 69.8	260.1 ± 83.2	258.8 ± 77.6
Weight of capsule contents per capsule (*mg*)	30.4 ± 10.1	29.2 ± 6.2	29.8 ± 11.4
Ratio of weight of capsule contents to the weight of the empty capsules	9.4 ± 4.4	9.9 ± 3.6	9.6 ± 3.7
Capsule diameter (*mm*)	6.9 ± 0.9	6.9 ± 1.1	6.9 ± 1.0
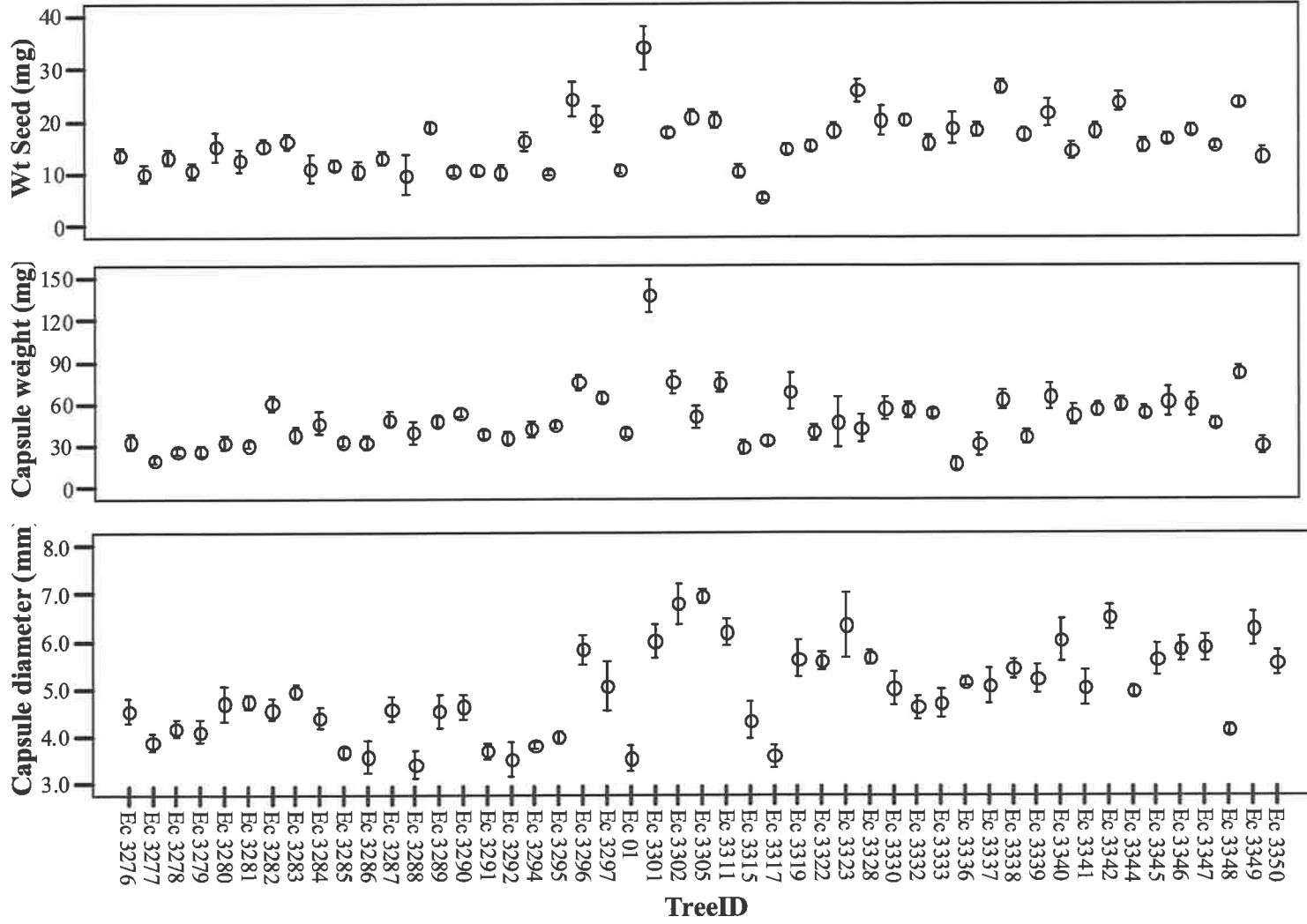
Figure 5.1: Mean (± 95% CI) weight of capsule contents, dry weight of capsules and capsule diameter for individual *E. camaldulensis* trees.
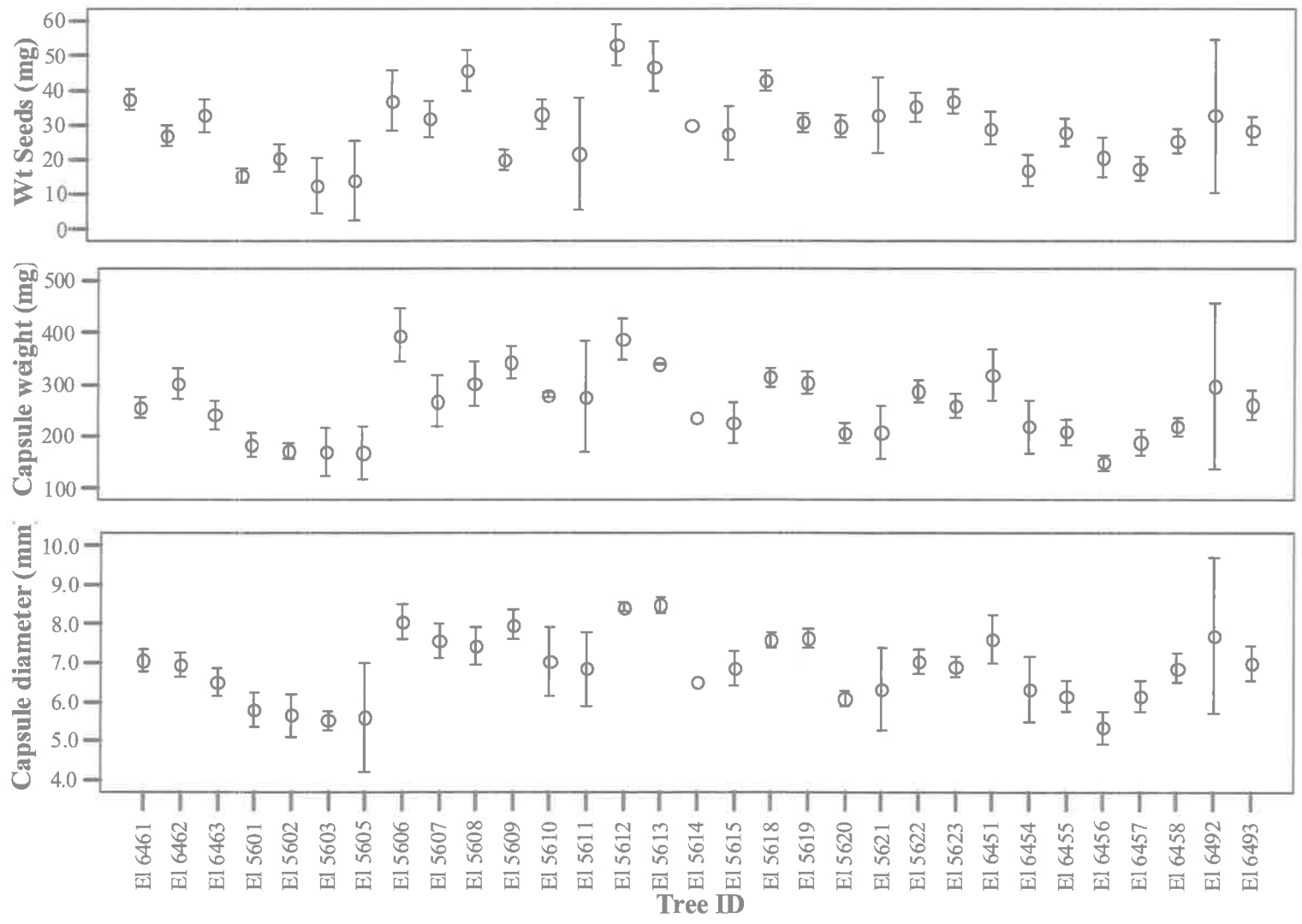
Figure 5.2: Mean (± 95% CI) weight of capsule contents, dry weight of capsules and capsule diameter for individual *E. leucoxylon* trees.

5.3.4.2   Within tree variation in seed and capsule measurements

Potentially, pollinators enter the tree canopy at different positions, for example birds tend to enter the upper canopy when making movements between trees (Hingston & Potts 2005). Pollinator behaviour may influence seed and capsule production through their influence on pollen deposition patterns. To test this, I compared the characteristics of capsules collected from different sections of the canopy (upper, mid, lower) (Table 5.7). Data were pooled across the two years for trees where capsules had been collected from all levels in the canopy (some trees were excluded because they were too high for fruits to be collected from the upper canopy). Fruits collected from the upper canopy had lower weight of capsule contents and lower weight of capsules (Table 5.7). The difference in weight of capsule contents was not significant (One-way ANOVA on $\log_{10}$ transformed data, $F_{2,328}=0.19$, $P=0.83$), but capsule weight was significantly lower in the upper canopy than the lower or mid canopy (One-way ANOVA on sqrt transformed data, $F_{2,292}=3.81$, $P=0.02$). However, when TreeID was taken into account, this relationship was not significant (Nested ANOVA, Canopy position $F_{3,81}=663.4$, $P=<0.001$; TreeID(Canopy position) $F_{75,217}=7.85$, $P=<0.001$). That is, the variation in capsule weight was due to variation between individual trees rather than due to the position in the canopy that they were collected from.

Capsules collected from the upper canopy of *E. leucoxylon* trees were smaller in diameter and contained fewer capsule contents than capsules collected from the lower and mid canopy (Table 5.7b). I performed One-way ANOVA on these data but due to the extremely small sample size for capsules collected from the upper canopy, I interpret the results with caution. There was no significant difference in the weight of capsule contents for fruits collected from different parts of the canopy ($F_{2,262}=1.14$, $P=0.32$), the weight of capsules (square-root transformed data, $F_{2,215}=0.54$, $P=0.59$) or the diameter of capsules ($F_{2,215}=0.47$, $P=0.63$). The ratio of capsule contents to capsule weight was significantly lower for capsules collected from the upper canopy compared to the lower and mid canopy ($F_{2,213}=2.81$, $P=0.06$). That is, capsules from the upper canopy contained more capsule contents for the size of the capsule than capsules from other parts of the canopy. If we assume that the difference in weight of capsule contents is due to a greater number of seeds, this may reflect a higher seed set for capsules in the upper canopy. However, again taking into account the effect of "Tree", nested ANOVA

showed that the difference was due to the tree rather than canopy position (Canopy position $F_{7,155}=171.6$, $P=<0.001$; TreeID(Canopy position) $F_{=137,82}=2.99$, $P=<0.001$).

Table 5.7: Characteristics of capsules (mean ± s.d.) collected from different positions in the canopy of *E. camaldulensis* and *E. leucoxylon* paddock trees.

n = number of seedlots. Capsule weight refers to the dry weight of capsules.

a. *E. camaldulensis*

Measurement	Low (*n*=141)	Mid (*n*=129)	Upper (*n*=61)
Weight of an empty capsule (*mg*)	43.7 ± 17.9	47.9 ± 21.2	39.0 ± 16.0
Weight of capsule contents per capsule (*mg*)	15.5 ± 5.5	16.0 ± 6.1	15.2 ± 5.2
Ratio of weight of capsule contents to the weight of the empty capsules	3.2 ± 1.4	3.3 ± 1.5	2.8 ± 1.3
Capsule diameter (*mm*)	4.7 ± 1.0	4.8 ± 0.9	4.8 ± 0.9

b. *E. leucoxylon*

Measurement	Low (*n*=171)	Mid (*n*=90)	Upper (*n*=4)
Weight of an empty capsule (*mg*)	255.5 ± 74.4	267.7 ± 81.9	257.4 ± 67.3
Weight of capsule contents per capsule (*mg*)	29.8 ± 11.1	30.4 ± 11.4	21.8 ± 10.2
Ratio of weight of capsule contents to the weight of the empty capsules	9.5 ± 3.5	9.2 ± 3.6	13.6 ± 6.2
Capsule diameter (*mm*)	6.9 ± 1.0	7.0 ± 1.0	6.7 ± 0.8

5.3.4.3   Tree physical characteristics and seed and capsule characteristics

To determine whether tree size or demography was correlated with seed production, data for seed and capsule characteristics were pooled across two years and averages calculated per tree.

There were no significant relationships between tree size variables and seed and capsule characteristics for *E. camaldulensis* trees (Table 5.8), but there were significant correlations between seed and capsule characteristics and tree isolation, with the exception of the ratio of weight of capsule contents to capsule weight. In all cases, the relationship was positive: more isolated trees produced larger and heavier capsules, as well as a greater weight of capsule contents. Because the weight of capsule contents represents the weight of both seed and chaff, it is impossible to know whether isolated trees are producing more seed (and are therefore unlikely to be pollination limited) or more chaff (potentially pollination-limited). However, because there was very little relationship between tree distance and the ratio of seed weight to capsule weight, isolated trees are not producing proportionally more capsule contents for the size of capsules.

For *E. leucoxylon* trees there was very little correlation between tree size and seed and capsule characteristics (Table 5.8), though there appeared to be a weak positive relationship between tree size and capsule diameter. Tree isolation was not significantly correlated with seed and capsule characteristics. Interestingly, in contrast to *E. camaldulensis*, relationships of tree size to seed and capsule characteristics were negative. More isolated trees produced a lower weight of capsule contents and smaller capsules.

Table 5.8: Pearson correlation coefficients (*r*) and significance (*p*, in parentheses) of seed and capsule characteristics of *E. camaldulensis* and *E. leucoxylon* trees with tree size and isolation.

Data for *E. camaldulensis* weight of capsule contents, weight of empty capsules and the ratio of weight of capsule contents to capsule weight were $\log_{10}$ transformed to satisfy assumptions of normality.

Parameter	Weight capsule contents	Weight empty capsule	Capsule diameter	Ratio capsule wt: seed wt
E. camaldulensis				
Tree Size	-0.05 (0.77)	0.09 (0.63)	0.15 (0.42)	0.20 (0.27)
Tree Isolation	0.48 (0.01)	0.45 (0.01)	0.63 (0.00)	0.03 (0.86)
E. leucoxylon				
Tree Size	0.03 (0.87)	0.18 (0.33)	0.31 (0.10)	0.12 (0.52)
Tree Isolation	-0.15 (0.43)	-0.20 (0.29)	-0.24 (0.20)	0.11 (0.57)

Table 5.9: Capsule characteristics (mean ± S.E.) of trees found at different slope positions, and results of one-way ANOVA (F) of seed and capsule variables with slope position.

Significance (*P*) of ANOVA in parentheses. Superscripts represent groups with significantly different means (*P*=0.05) as determined by Tukey's post hoc test.

a. E. camaldulensis

Slope position	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt: seed wt
Low (*n*=12)	13.4 ± 1.5	50.2 ± 5.7	4.7 ± 0.35	3.9 ± 0.34[b]
Mid (*n*=16)	17.1 ± 1.7	48.9 ± 6.7	4.8 ± 0.19	2.9 ± 0.23[a]
Upper (*n*=5)	15.8 ± 1.9	53.8 ± 8.2	4.8 ± 0.42	3.0 ± 0.28[a,b]
$F_{2,30}$	1.96 (0.16)	0.23 (0.79)	0.39 (0.68)	3.61 (0.03)

b. E. leucoxylon

Slope position	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt: seed wt
Low (*n*=3)	26.8 ± 3.2	209.0 ± 34.3[a]	6.4 ± 0.65	7.9 ± 0.26
Mid (*n*=17)	30.3 ± 2.7	282.3 ± 15.7[b]	7.2 ± 0.20	10.6 ± 0.80
Upper (*n*=10)	28.2 ± 3.0	228.9 ± 16.2[a]	6.5 ± 0.24	9.9 ± 0.89
$F_{2,27}$	0.23 (0.79)	3.57 (0.04)	2.74 (0.08)	1.40 (0.26)

5.3.4.4 Tree slope position and seed and capsule characteristics

For *E. camaldulensis* trees, the weight of capsule contents, the weight of capsules and capsule diameter did not vary significantly for trees found at different slope positions (Table 5.9a). However, trees occurring on lower slope positions had a significantly higher ratio of capsule weight to weight of capsule contents than trees found on mid and upper slopes; that is, trees found lower slope produced fewer capsule contents for the weight of capsules.

Eucalyptus leucoxylon trees found at mid slope positions had larger capsules and a greater weight of capsule contents than trees at lower and upper slope positions (Table 5.9b). One-way ANOVA showed a significant difference in the weight of capsule contents between trees at different slope positions ($P=0.04$). However, only a small number of *E. leucoxylon* trees were surveyed at lower slope positions and there was large variance associated with estimates of seed and capsule characteristics between the three trees, thus, the significance of this result is questionable. Further sampling would be required to test the association properly.

5.3.4.5 Seed production in intact woodland vegetation and paddock trees

I surveyed seed and capsule production for trees in natural (intact) vegetation and paddock trees for both species. A comparison of seed and capsule characteristics for *E. camaldulensis* trees found in natural intact woodland and isolated trees (Table 5.10a) showed that isolated trees tended to produce significantly heavier capsules than trees in natural vegetation ($P=0.02$). However, this did not translate to a greater production of capsule contents as trees in natural vegetation had a significantly lower ratio of capsule weight to seed weight ($P=0.01$). That is, for the smaller weight of the empty capsules, trees in natural vegetation had higher weight of capsule contents than isolated trees.

Eucalyptus leucoxylon trees found in natural vegetation tended to have a greater weight of capsule contents and heavier capsules than isolated trees, though these trends were not significant (Table 5.10b). For both *E. camaldulensis* and *E. leucoxylon*, trees found in natural vegetation tended to have a lower ratio of capsule weight to capsule contents, indicating that these trees are producing a greater amount of capsule contents for the size of the capsule.

Table 5.10: Mean (± S.E.) seed and capsule characteristics of *E. camaldulensis* and *E. leucoxylon* trees in natural vegetation and paddock trees, and results of t-test of seed and capsule characteristics against vegetation type.

Significance (*P*) in parentheses.

a. *E. camaldulensis*

Vegetation type	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt:seed wt
Natural (*n*=5)	12.9 ± 0.84	28.8 ± 2.3	4.4 ± 0.17	2.2 ± 0.09
Paddock (*n*=28)	16.3 ± 1.2	53.9 ± 4.2	4.9 ± 0.19	3.5 ± 0.20
t_{31}	-1.16 (0.26)	-2.44 (0.02)	-1.94 (0.07)	-2.61 (0.01)

[a] Equal variances not assumed

b. *E. leucoxylon*

Vegetation type	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt:seed wt
Natural (*n*=5)	32.3 ± 3.0	267.5 ± 18.3	6.8 ± 0.17	8.8 ± 1.6
Paddock (*n*=27)	28.9 ± 2.0	256.0 ± 13.1	6.9 ± 0.18	10.0 ± 0.6
t_{30}	-0.55 (0.59)	-0.28 (0.78)	0.06 (0.95)	0.64 (0.53)

Table 5.11: Mean (± S.E.) seed and capsule characteristics for *E. camaldulensis* and *E. leucoxylon* trees found in various tree density categories, and one-way ANOVA of capsule characteristics with tree density.

Significance (*P*) in parentheses. Superscripts represent groups with significantly different means (*P*=0.05) determined by a Tukey's post hoc test.

a. *E. camaldulensis*

Tree density	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt:seed wt
Natural (*n*=5)	12.9 ± 0.8[a]	28.8 ± 2.3[a]	4.4 ± 0.2[a]	2.2 ± 0.1[b]
High (*n*=5)	12.7 ± 1.6[a]	40.5 ± 3.5[a,b]	3.9 ± 0.3[a]	3.3 ± 0.3[a,b]
Medium (*n*=10)	13.8 ± 2.0[a]	50.1 ± 5.4[b]	4.5 ± 0.3[a]	4.0 ± 0.4[a]
Low (*n*=13)	19.7 ± 1.7[b]	62.0 ± 7.6[b]	5.6 ± 0.2[b]	3.2 ± 0.3[a,b]
$F_{3,29}$	3.62 (0.03)	3.67 (0.02)	8.42 (0.00)	3.72 (0.02)

b. *E. leucoxylon*

Tree density	Weight capsule contents (mg)	Weight empty capsule (mg)	Capsule diameter (mm)	Ratio capsule wt:seed wt
Natural (*n*=5)	32.3 ± 3.0	267.5 ± 18.3	6.8 ± 0.2	8.8 ± 1.6
High (*n*=4)	22.9 ± 2.3	191.5 ± 15.4	6.1 ± 0.3	8.9 ± 0.9
Medium (*n*=7)	34.9 ± 3.9	283.1 ± 24.0	7.1 ± 0.3	9.1 ± 1.2
Low (*n*=16)	27.8 ± 2.7	260.4 ± 17.3	6.9 ± 0.2	10.6 ± 0.8
$F_{3,28}$	1.57 (0.22)	1.92 (0.51)	1.26 (0.31)	0.70 (0.56)

5.3.4.6   Paddock tree density and seed production

Tree seed production may not necessarily simply be a function of the distance to the nearest conspecific, but may also be related to the number of local mating partners; so may be reliant on tree density rather than distance. Trees were characterised as occurring in natural, low, medium and high tree density situations (see methods). For *E. camaldulensis*, trees found in low-density situations had significantly greater weight of capsule contents ($P=0.03$) and larger diameter capsules ($P=<0.001$) than trees in other density categories (Table 5.11a). However, the ratio of capsule weight to seed weight for these trees was not significantly different to other paddock trees in different density categories or to trees in natural vegetation. *Eucalyptus camaldulensis* trees in medium density situations produced the smallest amount of capsule contents for the weight of empty capsules and trees in natural vegetation produced the greatest amount.

For *E. leucoxylon* trees, trees in high density situations produced the smallest capsules and had the smallest weight of capsule contents (Table 5.11b), with trees in low density situations also producing lightweight capsules and a low weight of capsule contents. Interestingly, as for *E. camaldulensis*, *E. leucoxylon* trees in natural vegetation produced the greatest weight of capsule contents for the weight of capsules. Low-density *E. leucoxylon* trees had the lowest weight of capsule contents to capsule weight. However, one-way ANOVA showed that these trends were not significant (Table 5.11).

5.3.5   Germination rates of *E. camaldulensis* and *E. leucoxylon* trees

5.3.5.1   Summary

Across the population of *E. camaldulensis* trees the mean number of seedlings germinated from 100mg of capsule contents was 42 in 2000 and 92 in 2002 (Table 5.12). A number of trees failed to produce any germinants in 2000 (Trees Ec3290, Ec3291, Ec3292, Ec3294, Ec3295 and Ec3296). By excluding trees with a zero germination rate the mean number of seedlings produced in 2000 was 42.4. In both years there was enormous variation in germination rate between seedlots and between individual trees (Figure 5.3). One-way ANOVA confirmed there was a significant difference in germination rate between individual trees ($F_{43,371}=20.1$, $P=<0.001$). A non-parametric Mann-Whitney U test (data on seedling numbers were non-normally distributed due to the high incidence of zero germination rates and data transforms were

unsuccessful) showed there was a significantly higher germination rate in 2002 than in 2000 ($Z = -10.15$ p<0.001).

Eucalyptus leucoxylon trees produced far fewer germinants per 100mg of capsule contents than *E. camaldulensis* trees and germination rates were much more consistent across the two years. *Eucalyptus leucoxylon* trees had an average germination rate of 13 seedlings in 2001 and 15 seedlings in 2003. Again, if trees with a zero germination rate had been excluded the average germination rate in 2001 would have been 17.1 and 18.7 in 2003. Germination rate varied significantly between individual trees ($F_{27,187} = 4.16$, $P = <0.001$) (Figure 5.3b). However, there was no significant difference in average germination rates between years ($t = -0.69$, $P = 0.49$).

5.3.5.2   *Capsule characteristics and germination rate*

Seedling and capsule variables for *E. camaldulensis* were non-normally distributed and data transformations were unsuccessful. Thus a Spearman's rank correlation coefficient (for non-parametric data) was calculated to look at the relationship between seed weight, capsule weight and capsule size and germination rate for individual seedlots. Germination rate was not correlated with the weight of capsule contents ($P = 0.22$) but was significantly positively associated with capsule weight ($P < 0.001$) (Table 5.13). However, germination rate was negatively correlated with capsule diameter ($P < 0.001$). Germination rate was significantly positively correlated with the ratio of capsule content weight to capsule weight ($P < 0.001$), which indicates that capsules with a higher proportion of capsule contents had a lower germination rate, perhaps indicating some competition between seeds for maternal resources.

Seedling data for *E. leucoxylon* were also non-normally distributed due to the high incidence of zeros. Spearman's rank correlation showed there were positive correlations between all capsule measurements and germination rate (Table 5.13). However, there was a negative relationship between germination rate and the ratio of capsule content weight to capsule weight, indicating that trees with a higher proportion of capsule contents per capsule weight had a higher germination rate.

Table 5.12: Average germination rates (the number of seedlings germinated from 100mg of capsule contents) for *E. camaldulensis* and *E. leucoxylon* trees.

	E. camaldulensis		*E. leucoxylon*	
	2000	**2002**	**2001**	**2003**
Total weight of capsule contents	27g	18g	8.6g	17g
Number of trees	38	35	23	29
Average weight capsule contents collected per tree	710mg	514mg	374mg	594mg
Total number of seedlings	8885	20977	1059	2646
Average no. seedlings germinated per 100mg of capsule contents ($\pm$ S.E)	33.4 $\pm$ 2.9	92.1 $\pm$ 6.0	13.0 $\pm$ 1.5	14.6 $\pm$ 1.2
Range	0 - 258	0 – 384	0 - 86	0 - 87

a. *E. camaldulensis*
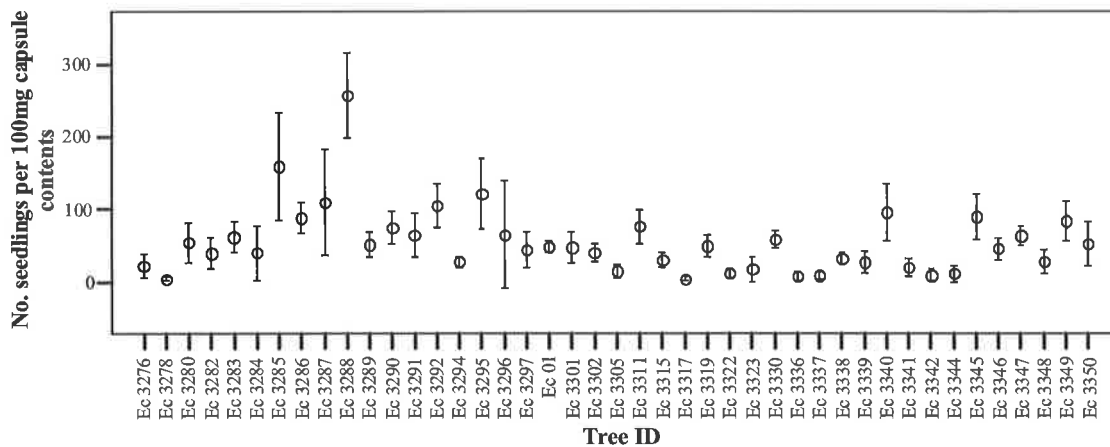
b. *E. leucoxylon*
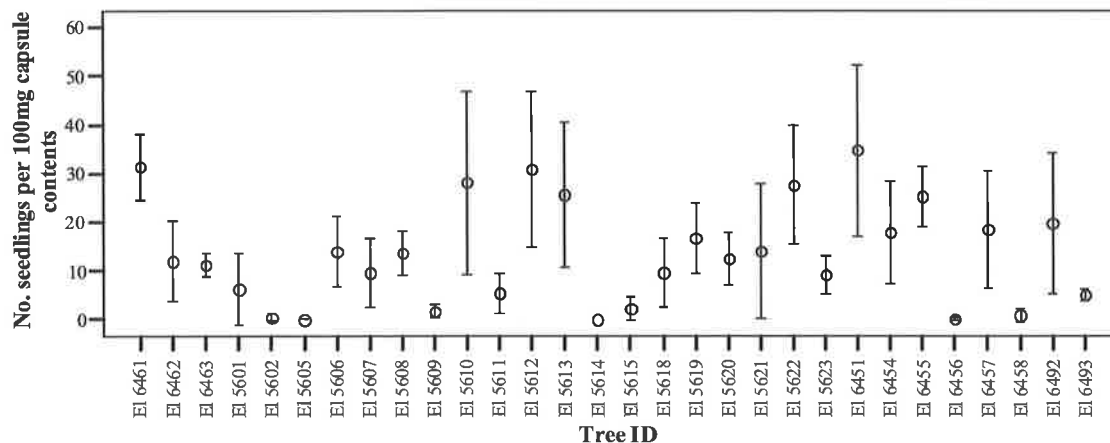
Figure 5.3: Mean ($\pm$ 95% CI) number of *E. camaldulensis* and *E. leucoxylon* seedlings germinated from 100mg of capsule contents, averaged across two flowering seasons.

5.3.5.3   Tree size and isolation versus germination rate

For *E. camaldulensis* trees, there was no significant correlation of tree size variables with germination rate in either year, though there was a negative trend in both years (Table 5.14). However, when the seedling data were averaged over the two years, tree size variables were significantly negatively correlated with germination rate. That is, larger trees on average produced fewer seedlings. There was very little relationship between germination rates and isolation distances in either years or when averaged (Table 5.14).

For *E. leucoxylon* trees, tree size was negatively associated with germination rate in each year, however this relationship was not significant. Interestingly, the degree of tree isolation was not significantly correlated with germination rate in 2001, but in 2003 and when data was averaged across the two years there was a moderately strong negative relationship between tree isolation and germination rate (though this was marginally non-significant). This trend indicates that more spatially isolated *E. leucoxylon* trees tend to have lower germination rates.

5.3.5.4   Canopy position and germination rate

In both *E. camaldulensis* and *E. leucoxylon* trees the average germination rate (averaged across 2 years) was lower for capsules collected from the upper canopy than those collected from the mid or lower canopy (Table 5.15). Nonparametric Kruskal-Wallis tests on ranked seedling data showed that significantly fewer seedlings were germinated from capsules collected from the upper canopy in *E. camaldulensis* trees, but this was not significant for *E. leucoxylon* trees (very small sample size was an issue). However, when TreeID was taken into account in a nested ANOVA, this relationship was not significant as there was significant variation associated with TreeID ($P<0.001$).

Table 5.13: Correlations (ρ) of germination rate and seed and capsule characteristics of *E. camaldulensis* and *E. leucoxylon* trees.

Spearman's rank correlation (ρ) and significance (*P*) in parentheses.

Germination rate	Weight capsule contents	Weight empty capsule	Capsule diameter	Ratio capsule wt:seed wt
E. camaldulensis	-0.06 (0.22)	0.20 (0.00)	-0.18 (0.00)	0.24 (0.00)
E. leucoxylon	0.41 (0.00)	0.35 (0.00)	0.25 (0.00)	-0.11 (0.11)

Table 5.14: Correlations (*r*) of germination rate and tree size and distance characteristics of *E. camaldulensis* and *E. leucoxylon* trees.

All seedling variables sqrt(*x*+0.5) transformed to satisfy assumptions of normality. Significance (*P*) in parentheses.

a. *E. camaldulensis*

Parameter	Seedlings 2000	Seedlings 2002	Average 2000-2002
Tree Size	-0.30 (0.15)	-0.34 (0.09)	-0.48 (0.01)
Tree Isolation	0.32 (0.12)	-0.28 (0.15)	-0.08 (0.67)

b. *E. leucoxylon*

Parameter	Seedlings 2001	Seedlings 2003	Average 2001-03
Tree Size	-0.27 (0.22)	-0.12 (0.55)	-0.19 (0.33)
Tree Isolation	-0.11 (0.64)	-0.36 (0.06)	-0.34 (0.06)

Table 5.15: Mean (± S.E.) number of germinants per 100mg capsule contents for capsules collected from different positions in the canopy of *E. camaldulensis* and *E. leucoxylon* paddock trees.

Number of seedlots in parentheses. Results of non-parametric Kruskal-Wallis test (X^2) for significant differences between groups (superscripts represent significant differences between group means at *P*=0.05).

Canopy Position	*E. camaldulensis*	*E. leucoxylon*
Low	56.2 ± 60.4 [a] (170)	15.7 ± 1.3 (177)
Mid	59.7 ± 63.8 [a] (157)	12.1 ± 1.4 (84)
Upper	33.1 ± 40.8 [b] (54)	2.2 ± 1.8 (4)
Kruskal-Wallis X^2	11.576	4.711
P	0.003	0.095

5.3.5.5   Slope position and germination rate

In both *E. camaldulensis* and *E. leucoxylon* trees, trees located on upper slopes had a lower germination rate than trees found at other slope positions, with trees found at low slope positions having the highest germination rate (Table 5.16). However, these differences were not statistically significant.

5.3.5.6   Germination rate in intact vegetation and paddock trees

There was a different pattern for the average germination rate of trees in natural vegetation and isolated trees for *E. camaldulensis* and *E. leucoxylon*. In *E. camaldulensis*, trees found in natural vegetation had a lower germination rate than isolated trees and, in *E. leucoxylon,* trees in natural vegetation had a greater germination rate than isolated trees (Table 5.17). However, overall there was no significant difference in germination rate between the two vegetation types for either species. Interestingly, the variance in estimates of germination rate was greater for *E. camaldulensis* than *E. leucoxylon* trees for both vegetation categories.

5.3.5.7   Paddock tree density and germination rate

Eucalyptus camaldulensis paddock trees found at high density had the highest germination rate, while low density trees were intermediate (Table 5.18). Interestingly, medium density paddock trees had a lower germination rate than trees found in natural vegetation. However, one-way ANOVA showed that high density paddock trees had a significantly higher germination rate than all other density categories, suggesting that in the paddock tree environment conspecific density may be an important determinant of reproductive success.

For *E. leucoxylon* trees, trees found in natural vegetation had the highest germination rate and high density paddock trees had the lowest, though there was no significant difference in germination rates across the trees sampled. This suggests that, for the bird-pollinated species, *E. leucoxylon*, conspecific density does not have a significant impact on seed production. In contrast to *E. camaldulensis*, medium density trees had the highest germination rate.

Table 5.16: Mean (± S.E.) number of germinants from 100mg capsule contents for *E. camaldulensis* and *E. leucoxylon* paddock trees located at different slope positions.

One-way ANOVA (F) on square root transformed seedling data and significance (*P*) in parentheses. *n* = number of trees in each category.

Slope position	*E. camaldulensis*	*E. leucoxylon*
Low	91.6 ± 23.0 (*n=11*)	13.5 ± 6.7 (*n=3*)
Mid	55.3 ± 7.4 (*n=14*)	13.2 ± 2.7 (*n=17*)
Upper	49.3 ± 13.6 (*n=5*)	10.2 ± 2.5 (*n=10*)
$F_{2,28}$	1.16 (0.33)	0.39 (0.68)

Table 5.17: Mean (± S.E.) number of germinants from 100mg capsule contents of *E. camaldulensis* and *E. leucoxylon* trees in natural vegetation and paddock trees.

Results of t-test and significance (*P*) in parentheses. *n* = number of trees in each category.

Vegetation Type	*E. camaldulensis*	*E. leucoxylon*
Natural	49.0 ± 21.1 (*n=5*)	19.3 ± 4.3 (*n=5*)
Paddock	69.1 ± 10.1 (*n=28*)	11.8 ± 1.9 (*n=27*)
t_{32}	-0.69 (0.49)	-1.26 (0.22)

Table 5.18: Mean (± S.E.) number of germinants from 100mg capsule contents of *E. camaldulensis* and *E. leucoxylon* trees at different levels of conspecific density.

One-way ANOVA on square root transformed seedling data and significance (*P*) in parentheses. *n* = number of trees in each category. Superscripts represent groups with significantly different means (*P=0.05*) as determined by a Tukey's post-hoc test.

Tree Density	*E. camaldulensis*	*E. leucoxylon*
Natural	49.0 ± 21.1 [a] (*n=5*)	19.3 ± 4.3 (*n=5*)
High	143.7 ± 37.0 [b] (*n=5*)	9.5 ± 5.0 (*n=4*)
Medium	39.1 ± 8.5 [a] (*n=10*)	16.8 ± 3.5 (*n=7*)
Low	63.6 ± 7.6 [a] (*n=13*)	10.1 ± 2.5 (*n=16*)
$F_{3,32}$	7.54 (0.00)	1.41 (0.26)

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5.4 Discussion

Isolated paddock trees are often referred to as the "living dead" (Janzen 1986), persisting in the landscape by virtue of their longevity but contributing little to population processes and/or maintenance. In a survey of reproductive effort of a sample of *E. camaldulensis* and *E. leucoxylon* paddock trees I found that in both species the great majority of trees (60-70%) had high levels of floral production and 30-40% of trees had high levels of fruit production. Over the two years surveyed only a single *E. camaldulensis* tree and three *E. leucoxylon* trees failed to produce any visible fruits. On a coarse scale, this indicates that the majority of paddock trees are indeed reproductively viable. For both species, 50-60% of trees with high levels of bud production also had high capsule production. While both species are self-compatible, previous research has indicated only moderate levels of self-compatibility and thus, these results are initially suggestive that paddock trees are receiving sufficient pollination services. I had predicted that paddock trees of the insect-pollinated species, *E. camaldulensis*, might suffer the effects of spatial isolation more severely than the bird-pollinated species, *E. leucoxylon*. In this survey I found that patterns of reproductive output were very similar between the two species – approximately the same proportion of trees were noted to have high and moderate fruit production in both species. In addition, fruit set in *E. camaldulensis* paddock trees was relatively high (~40%) while in the limited number of *E. leucoxylon* trees surveyed fruit set was low (~10%).

All measures of flower and fruit production reflected differences in reproductive strategy between the two species. *Eucalyptus camaldulensis* produces numerous small flowers and fruits per leaf unit and many small seeds per fruit, and consequently, the number of seedlings germinated from capsules was high (up to 390 seedlings per 100mg capsule contents). In contrast, *E. leucoxylon* produces few, large flowers and fruits with a smaller number of seeds per fruit and thus a lower number of seedlings were germinated from capsules (~10-20 per 100mg capsule contents) than *E. camaldulensis*. However, for both species, measures of flower, fruit and seed production and germination rates varied greatly between individual paddock trees. While this, to a certain extent, may reflect the genetic differences between individuals, the large variation in measures of reproductive output for individual trees may also be due to a

whole range of demographic and ecological factors. I attempted to measure a number of these variables and these will be discussed below.

5.4.1   Tree size

In the previous chapter, I found that the majority of paddock trees are large in size, both in terms of girth and canopy spread. In addition, in the survey of reproductive effort a high proportion of paddock trees had high floral production, indicating that these trees represent a substantial floral resource for pollinators. Previous studies have shown that pollinators respond to plants with a large floral display by increasing visitation rates, but visiting a smaller proportion of flowers per plant (Klinkhamer *et al.* 1989; Klinkhamer & de Jong 1990; Grindeland *et al.* 2005). This behaviour is likely to provide maximal opportunity for transfer of outcrossed pollen and to limit the rate of geitonogamous selfing, and for plants such as eucalypts this should be reflected in increased fruit production. Besides the influence of plant size on pollinator behaviour, plant size is also a potential indicator of age, in which case the population of paddock trees sampled here is highly skewed towards older individuals (Chapter 4). Potentially, larger individuals may be experiencing age-related decline in fecundity.

Measures of plant size were not correlated with flower or capsule production in either species, though there was a weak negative association of plant size with the number of buds per umbel in *E. camaldulensis*, potentially indicating a decline in fecundity for larger, older individuals in this species. Interestingly though the opposite trend was observed in *E. leucoxylon*, with a weak positive association between plant size and the number of flowers and number of capsules per inflorescence. Similarly, there was very little association between measures of plant size and seed and capsule characteristics (weight of capsule contents, weight and size of capsules) for either species. However, there was a weak positive association between the ratio of capsule weight to capsule content weight for both species, indicating that large trees produce a lower weight of capsule contents for the weight of capsules. While this may indicate a greater investment by trees in the weight of capsules in order to protect seeds, it may also indicate that these trees are receiving inadequate pollination and producing fewer seeds per capsule.

Plant size was significantly negatively correlated with germination rate in *E. camaldulensis* when data from the two years were pooled. A similar negative trend was observed for *E. leucoxylon* trees though this was not statistically significant. I had predicated that larger trees might receive more pollinator visits and therefore more outcrossed pollen, reflected in a higher germination rate. However, the opposite appears to be the case. Since the number of germinants was based on 100mg capsule contents, it appears that larger trees potentially have a higher proportion of chaff. Chaff is essentially unfertilised ovules, which indicates perhaps these trees are indeed receiving fewer pollinator visits. Alternatively, a lower germination rate may reflect increased receipt of selfed pollen, due to increased pollinator bout lengths on larger trees or if the rate of outcrossed pollen carryover is low. Increased geitonogamous selfing could have potentially led to inbreeding depression in the form of embryological failure (reflected in a reduction in the number of seeds) and/or reduced seed viability (reflected in a reduction in the germination rate). Nevertheless, if indeed large trees are old aged, then a lower germination rate may also reflect a reduced ability of large, old trees to apportion resources to seeds.

5.4.2   Tree spatial isolation

The degree of spatial isolation of a tree can impact on a number of factors relating to reproductive effort and output. The distance to nearest neighbour (nearest neighbour of any eucalypt species) indicates the degree of competition a tree may have for resources, and thus the resources it has available to allocate to reproduction. The distance to nearest conspecific (nearest neighbour of same species) may indicate the probability of reproductive success assuming that pollinator visitation rates are higher for closely-spaced trees. Research has also shown that spatially isolated plants are likely to receive fewer pollinator visits and that pollinators increase their bout lengths on isolated plants, increasing the probability of geitonogamous selfing (Pyke 1979; de Jong *et al.* 1992; Goulson 2000).

There were no significant correlations between tree spatial isolation and bud and capsule production in either species, though there was a weak positive association between distance and the number of capsules per leaf unit in *E. camaldulensis* and a weak negative association in *E. leucoxylon*. It is difficult to tell whether these trends are due to pollination or competition effects. A higher rate of capsule production in isolated

trees (e.g. *E. camaldulensis*) may reflect an enhanced ability of isolated trees to capture resources and allocate them to reproduction due to reduced competition from other individuals. A lower rate of capsule production in isolated plants (e.g. *E. leucoxylon*) may indeed reflect reduced pollinator visits or inbreeding depression due to increased selfing.

Interestingly, there were significant positive correlations between tree isolation and seed and capsule weight and size in *E. camaldulensis*. Isolated trees produced bigger capsules and a greater weight of capsule contents, but not a greater proportion of capsule contents to the weight of the capsule. This tends to indicate isolated trees have increased resources to invest in capsules – can produce bigger, heavier capsules to protect seeds. In contrast, *E. leucoxylon* showed negative associations between capsule characteristics and tree isolation. This may suggest that isolated *E. leucoxylon* trees may be showing signs of stress caused by tree isolation and are unable to allocate as many resources to reproduction as less stressed trees.

The degree of tree spatial isolation showed no association with germination rate for *E. camaldulensis* paddock trees, however there was a marginally non-significant negative correlation between tree isolation and germination rate for *E. leucoxylon*. Surprisingly this trend indicates that *E. leucoxylon* isolated trees, a bird-pollinated species, may be receiving fewer pollinator visits and/or increased selfing than the insect-pollinated species, *E. camaldulensis*. As *E. leucoxylon* isolated trees tend to produce smaller capsules, it can be assumed that they also produce smaller flowers which perhaps reduces their attractiveness to bird pollinators, leading to fewer pollinator visits. Alternatively, the degree of selfing experienced by isolated trees may be related to differences in pollinator behaviour driven by the energetic requirements of insect and bird pollinators. The small physical size of insect pollinators means that visiting only a small proportion of the canopy of these trees may easily satisfy their energetic needs. Even if they increase their foraging time on isolated trees this will only lead to increased selfing in a small proportion of the canopy. In contrast, large bird pollinators (e.g. wattlebirds, rainbow lorikeets, New Holland honeyeaters) must visit a large number of flowers per tree to satisfy their requirements, potentially increasing the selfing rate over a large proportion of the canopy.

5.4.3   Slope position

In the previous chapter I found that *E. camaldulensis* trees located on upper slopes were stressed and had a low proportion of intact canopy, which is likely to affect the reproductive output of these trees. Trees on upper slopes are likely exposed to high wind velocities and drying conditions, especially when combined with low soil moisture content and low nutrient content. In contrast, trees at lower slope positions may occur on deeper, more fertile and moist soils, but they may be exposed to salinity or increased soil acidity, common land degradation problems in the Mt Lofty Ranges.

Eucalyptus camaldulensis trees found on upper slopes had a high ratio of floral units to leaf units and a significantly greater number of capsules to leaf units than trees found on mid or lower slopes. As mentioned previously these trees had a lower proportion of intact canopy and were visibly stressed, thus this higher reproductive effort may indicate that these trees are investing more in reproduction to ensure that they contribute genetic material to the next generation before senescing. Capsule and seed weights did not differ between *E. camaldulensis* trees located at different positions in the landscape, however, lower slope trees had a significantly lower weight of capsule contents to the weight of capsules, but also the highest germination rate (though this was not significant). As *E. camaldulensis* is naturally adapted to high soil water conditions this may indicate that lower slope trees are able to invest more in producing heavier capsules to protect seeds and to apportion resources to seeds to ensure germinability. In *E. leucoxylon* trees, trees found mid slope had significantly heavier capsules than trees on lower and upper slopes, though they did not produce proportionally more seeds for capsules than other trees and germination rate did not differ from other trees.

5.4.4   Trees in natural vegetation versus paddock trees

It would seem reasonable to assume that pollinators would be more abundant in natural (undisturbed) vegetation than in the paddock tree environment as the natural vegetation system may provide greater number of resources and a greater variation of resources for pollinators (e.g. pollen and nectar sources, nesting habitat etc). As such it would be expected that trees in natural vegetation would have adequate pollination services and thus have high levels of fruit set in comparison to paddock trees. *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees indeed had a lower weight of capsule

contents to the weight of capsules than trees in natural vegetation (though this was only statistically significant for *E. camaldulensis*), potentially indicating lower pollination levels for isolated trees. However, the germination rate was not statistically different for trees in natural vegetation and paddock trees for either species. In fact, *E. camaldulensis* paddock trees had a higher average germination rate than trees in natural vegetation.

5.4.5   Average conspecific density

Local plant density is likely to affect plant reproductive success through its effects on mate availability and pollinator behaviour (Bosch & Waser 1999; Kirchner *et al.* 2005). In *E. camaldulensis* there was significant variation in seed and capsule weights between low, medium and high density trees, with low density trees producing significantly larger capsules and a greater weight of capsule contents than other trees. However, low density trees did not produce a greater weight of capsule contents for the weight of capsules compared to trees in natural vegetation or high density paddock trees, which tends to indicate that these trees are receiving adequate pollination despite extremely low conspecific densities. Germination rate was not significantly different for *E. camaldulensis* trees in natural vegetation and low and medium density paddock trees, but high density trees had a significantly higher germination rate than all other trees. High density *E. camaldulensis* paddock trees produced almost three times as many germinants per mass of capsule contents as trees in natural vegetation and more than twice as many as low density trees, suggesting that conspecific density has a big influence on fertilisation success of paddock trees.

For *E. leucoxylon* trees there was no significant effect of tree density on seed and capsule weights though trees with a low density of mating partners had the lowest ratio of capsule contents to capsule weight suggesting these trees produced a lower number of seeds. Low density trees indeed had a low germination rate, though trees found in high density groupings had the lowest germination rate. However, there was no statistically significant difference in germination rate between all density categories.

The striking difference in germination rate between high density *E. camaldulensis* trees and high density *E. leucoxylon* trees may reflect differing responses of pollinators to changes in plant density. Insect pollinators of *E. camaldulensis* may be behaving as

predicted by models of optimal foraging (Goulson 2000), increasing their visitation rate to plants in high density groupings but visiting fewer flowers per plant, facilitating higher outcrossing rates, reflected in a higher germination rate for these trees. In contrast, bird pollinators may respond to high density groupings of plants by establishing territories (e.g. Smith-Ramirez & Armesto 2003), increasing the within tree foraging rate and leading to increased selfing for the plant.

5.4.6   Overall summary

In this chapter I investigated a number of aspects of the reproductive ecology of *E. camaldulensis* and *E. leucoxylon* paddock trees. There were few clear associations between the demographic variables measured and flowering and fruiting characteristics. Larger trees appeared to suffer a decline in fecundity (smaller weight of capsule contents to the weight of empty capsules and lower germination rate), which may be age-related as it is in contrast to expectations that larger plants should receive more outcrossed pollen and experience less pollinator-mediated selfing (Klinkhamer *et al.* 1989; Klinkhamer & de Jong 1990; Grindeland *et al.* 2005). Despite the expectation that the degree of tree isolation (distance to nearest neighbour and distance to nearest conspecific) would impact on fruit and seed production, especially for the insect pollinated species, I observed no effect of tree isolation on fruit and seed production in *E. camaldulensis* trees. However, increasing spatial isolation of *E. leucoxylon* paddock trees tended to lead to a lower, though statistically non-significant, germination rate. It is unclear whether this was due to a lack of pollinator visits or the result of increased geitonogamous selfing. Nevertheless it is interesting that even highly dispersed trees of each species are receiving sufficient pollinator visits to set fruits and that their seeds are viable. This tends to indicate that the distances between paddock trees are still within the foraging range of both insect and bird pollinators.

Burrows (1995; 2000) found that isolated trees of *E. albens* and *E. melliodora* had significantly lower fecundity than trees found in intact natural vegetation. Paddock trees of both *E. camaldulensis* and *E. leucoxylon* appeared to produce fewer seeds (as a ratio of capsule contents to capsule weight) than trees in natural vegetation, however, germination rate did not differ significantly between paddock trees and trees in natural vegetation for either species, indicating that seed set was equivalent in both vegetation types. An interesting pattern emerges though when looking at the effect of local

conspecific density on germination rate. As expected for a species with a mobile pollinator, *E. leucoxylon* showed no difference in germination rate across all density categories, which indicates that bird pollinator foraging behaviour is maintained even in low density arrays of trees. I had initially predicted that a species with a less mobile pollinator might experience reduced seed set in low density arrays as a consequence of reduced pollinator visitation or increased geitonogamous selfing. Interestingly, in contrast to expectations, germination rates in the insect-pollinated *E. camaldulensis* were not different between trees found in low and medium density arrays and trees in natural vegetation, which again indicates that insect pollinator activity is sufficient even in very low density arrays of trees. Local conspecific density did indeed appear to impact on seed set in *E. camaldulensis* but not in the way predicted. The germination rate of high density paddock trees (trees at the same density as in natural vegetation but lacking understorey vegetation) was almost three times that of trees in natural vegetation, indicating that insect pollinators are indeed responding to plant density. In addition, it appears that insect foraging behaviour is also modified by patch composition. The lack of understorey vegetation may act to increase the density of insect pollinators on *E. camaldulensis* trees, increasing the rates of pollen transfer.

A reason for the lack of effect of spatial isolation on fruit production in *E. camaldulensis* paddock trees may be the presence of the introduced European honeybee, *Apis mellifera*. *Eucalyptus camaldulensis* trees are used for honey production in areas of South Australia (Paton 1996) and *Apis mellifera* is a frequent and abundant floral visitor of *E. camaldulensis* paddock trees (K. Ottewell pers. obs.), making up to 80% of all floral visits and taking both nectar and pollen. Honeybees are larger and more robust than native bees and are known to frequently make foraging forays of 1-1.5km (Waddington *et al.* 1994) and even up to 9.5km (Beekman & Ratnieks 2000). Several studies have shown no effect of habitat fragmentation on fruit set in the presence of introduced honeybees (Aizen & Feinsinger 1994a; Dick 2001). In Aizen and Feinsinger's study of the impacts of habitat fragmentation on 16 plant species in the Chaco Serrano, plant species that were heavily visited by Africanised honeybees showed no effect of fragmentation on fruit set, whereas other species in the same ecosystem not visited by honeybees had significant negative effects (Aizen & Feinsinger 1994a). Potentially, *E. camaldulensis* paddock trees may not be severely

affected by spatial isolation as the distances between trees are well within the foraging range of *Apis mellifera*.

Overall though, it appears that flowering and fruiting patterns of paddock trees are complex, resulting in high variability in measures of fruit and seed production between individual trees and little correlation between the demographic variables that I measured and reproductive characteristics. While a certain proportion of variability between individuals may be due to intrinsic genetic differences between individuals (especially given the potential genetic diversity of these trees, as described in Chapter 2), it is possible that one of the impacts of habitat clearance on reproductive patterns of paddock trees is, in fact, increased variability between individuals. Following vegetation clearance, each individual is exposed to a unique set of conditions (e.g. distance to nearest conspecific, local tree density, wind exposure, land salinisation etc) that may impact on their ability to produce flowers and fruit and how attractive they are to pollinators. However, it would have been necessary to compare a matched sample of trees in natural vegetation and paddock trees to test this hypothesis and this was not possible with the number of trees included in this study.

As mentioned, I was unable to find many significant correlations between the demographic variables that I measured and reproductive characteristics. In essence, I was testing for linear associations between individual variables, but it may have been more appropriate to take a multivariate modelling approach to determine which *set* of conditions best determine reproductive success. However, given the large amount of variation detected in this study it would have been necessary to greatly increase sample sizes to achieve any statistical power for this type of approach. In addition, there may have been a myriad of environmental variables that contribute to reproductive patterns that were not measured in this study. For future work I think it would be interesting to try to quantify the total resource value (floral display, nectar production) of each paddock tree, which would vary depending on topography, soil, tree health, current weather conditions, reproductive performance in previous year etc, and how this relates to pollinator behaviour in a highly dispersed population. While this may be easy to achieve for small plants that produce a small number of flowers or inflorescences per plant, it is difficult to accurately estimate total production in large *Eucalyptus* trees as both species produce many tens of thousands of flowers per tree.

Chapter 6   Mating system analysis of *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees

6.1   Introduction

In plants, the mating system determines the movement of genes in space and the mode of transmission of genes from one generation to the next (Barrett 2003). Most plants are hermaphroditic and many are capable of selfing as well as outcrossing. As such, plant mating systems are characterised by the frequency of selfing (s) or it's complement, the frequency of outcrossing ($t=1-s$; Brown (1990). Genetic information from the progeny of parental trees not only allows us to determine whether the progeny originated from outcrossing, selfing or apomixis, but amongst outcrossed progeny, it is also possible to estimate the degree of biparental inbreeding (mating with genetically related individuals), how often progeny are full sibs and the number of male parents represented in the seed crop (Ritland & Jain 1981; Ritland 2002). The frequency of selfing or outcrossing of individuals and populations has important implications for plant fitness (through inbreeding and heterosis) and population genetic structure (through the distribution of genetic diversity within and between populations) (Barrett & Hardner 1996). A plant's mating system may also determine its susceptibility to the consequences of habitat fragmentation, mediated by pollinator responses (as described in Chapter 5). For self-compatible species, it is predicted that habitat fragmentation will lead to increased inbreeding (through geitonogamous selfing or consanguineous mating) (Charlesworth & Charlesworth 1987) and self-incompatible species may experience fecundity declines through reduced mate availability or pollen discounting (Ritland 1991; Young *et al.* 1996).

6.1.1   Plant mating system responses to habitat fragmentation

Two of the most significant outcomes of the process of habitat fragmentation for plant populations are the reduction in plant density (on a local scale, through the loss of individual plants within a patch or across the landscape, the loss of whole tracts of vegetation) and changes to the spatial configuration of plant populations (e.g. trees restricted to small fragments in the landscape, increased distance between individuals). In many natural plant populations outcrossing rate has been shown to be positively correlated with plant density (Murawski & Hamrick 1991; van Treuren *et al.* 1993;

Raijmann *et al.* 1994; Hardner *et al.* 1996; Hodgins & Barrett 2006) and negatively associated with plant isolation (Aldrich & Hamrick 1998; Cascante *et al.* 2002; Fuchs *et al.* 2003).

A reduction in plant density and/or an increase in plant isolation frequently leads to declining levels of pollinator visitation or changes in pollinator foraging (for example, if the isolation distance is greater than the foraging range of pollinators) that has the effect of reducing the amount of outcrossed pollen received by plants or an increase in transfer of selfed pollen (Agren 1996; Collevatti *et al.* 2001; Mustajarvi *et al.* 2001). For example, trees in selectively logged tropical forest typically occur at one-tenth the density in unlogged forest and studies have found outcrossing rates of *Carapa procera* and *Shorea megistaphylla* were significantly lower in selectively logged patches compared to unlogged forests (Murawski *et al.* 1994; Doligez & Joly 1997).

Paddock trees experience extreme changes in plant density and population demography and several authors have reported that isolated trees included in their studies failed to set fruit or failed to produce any outcrossed progeny (Murawski & Hamrick 1991; Gribel *et al.* 1999; Butcher *et al.* 2005). Others have detected significant increases in the selfing rate of isolated trees in comparison to trees in continuous forest (*Pachira quinata* t_m = 0.78 and 0.92, respectively (Fuchs *et al.* 2003); *Symphonia globulifera* t_m = 0.74 and 0.90, respectively (Aldrich & Hamrick 1998); *Dinizia excelsa* t_m = 0.85 and 0.94, respectively (Dick 2001); *Samanea saman* t_m = 0.91 and 0.99, respectively (Cascante *et al.* 2002)). However, one study found that outcrossing rates of isolated trees were not significantly different to trees in continuous forest (*Enterolobium cyclocarpum*, t_m = 0.99 and 1.0, respectively (Rocha & Aguilar 2001).

Furthermore, the reduction in adult tree density also represents a reduction in the number of potential mating partners available to an individual, potentially leading to correlated matings between only a few individuals (Smouse & Sork 2004). Spatial isolation is also expected to lead to an increase in the rate of correlated paternity, as pollinators may be more likely to move between the closest trees rather than more distant trees. Indeed, Murawski & Hamrick (1991) found that trees at low adult densities tended to mate with fewer pollen donors than trees at high adult densities and Fuchs *et al.* (2003) found that isolated trees of *Pachira quinata* had an extremely high

level of correlated paternity compared to trees in continuous forest (r_p = 0.74 and 0.47, respectively). However, Wang *et al.* (2007) found that more isolated trees of *Eurycorymbus cavaleriei* had a greater number of pollen donors than trees in close proximity to other individuals.

6.1.2 Eucalypt mating systems

Mating system studies in the genus *Eucalyptus* typically indicate a mating system of predominant outcrossing with a significant amount of selfing (Moran & Bell 1983). The mating systems of *Eucalyptus camaldulensis* and *E. leucoxylon* have both been genetically characterised and indicate high levels of outcrossing (*E. camaldulensis* t_m=0.75 (Moncur *et al.* 1995); *E. leucoxylon* t_m=0.83 (Ellis & Sedgley 1993)), typical of widespread eucalypt species under natural conditions (e.g. *E. grandis* t_m=0.84 (Moran & Bell 1983), *E. delegatensis* t_m=0.77 (Moran & Brown 1980), *E. urophylla* t_m=0.91 (House & Bell 1994)). Within populations though, individual outcrossing rates can vary greatly (Brown *et al.* 1975; Griffin *et al.* 1987; Ellis & Sedgley 1993) and may even vary across the canopy of individual trees (Eldridge 1970; Hingston & Potts 2005). Outcrossing rates have been shown to be dependent on stand density for two eucalypt species (Sampson *et al.* 1995; Hardner *et al.* 1996) as the number and density of flowering trees determines the attractiveness of populations to pollinators.

However, other aspects of eucalypt mating systems have been less well characterised. In plants, the apparent selfing rate (*s*) also includes the results of consanguineous matings (i.e. mating with close relatives) (Ritland 2002). Such matings among related individuals ("biparental inbreeding") can arise from spatial sub-structuring of genotypes in a population (due to limited dispersal of seed or pollen) or from temporal variation in synchrony of mating among individuals and can result in substantial inbreeding depression. Biparental inbreeding is suggested to occur in eucalypt populations due to spatial structuring of genotypes (Moran *et al.* 1989; Hardner *et al.* 1998). However, only a few studies have evaluated levels of biparental inbreeding in *Eucalyptus*, and they show that levels of biparental inbreeding (t_m-t_s) are typically low to moderate (e.g. *E. cladocalyx* t_m-t_s=0.05 (McDonald *et al.* 2003); *E. marginata* t_m-t_s=0.11 (Millar *et al.* 2000); *E. delegatensis* t_m-t_s=0.02 (Moran & Brown 1980); *E. benthamii* t_m-t_s=0.10-0.17 (Butcher *et al.* 2005)).

The level of correlated paternity (r_p) (i.e. the probability that any two outcrossed offspring have the same paternal parent) amongst sibships can also be estimated from genetic data (Ritland 2002). There are few eucalypt studies that report correlated paternity results but those that do have shown highly variable results. For example, Sampson (1998) found the correlation of paternity in *E. rameliana* was 0.09, while *E. marginata* had extremely high levels of correlated paternity (r_p=0.75, Millar *et al.* 2000) and *E. benthamii* had moderate levels ($r_p = 0.36$, Butcher *et al.* 2005).

6.1.3   Aims

Evidence from previous studies suggests that both *E. camaldulensis* and *E. leucoxylon* maintain relatively high outcrossing rates in natural populations. Like other eucalypts neither species have specialised flowers and both achieve pollination via a range of animal vectors, primarily insects for *E. camaldulensis* and primarily birds for *E. leucoxylon*. In this chapter I use genetic data from progeny arrays to characterise the realised mating system of *E. camaldulensis* and *E. leucoxylon* paddock trees to determine how trees may be affected by reduced conspecific density and spatial isolation. Analyses were performed across two flowering seasons for each species to describe year-to-year variation in mating patterns. In addition, I explore the relationship of tree mating patterns with some of the demographic and reproductive characteristics of paddock trees.

6.2   Methods

6.2.1   Sampling

6.2.1.1   Adults

Mating system analyses were conducted on 46 adult *E. camaldulensis* and 28 adult *E. leucoxylon* paddock trees that had been included in previous surveys of tree demography (Chapter 4) and flowering and fruiting (Chapter 5). Paddock trees were located on aerial photographs and local tree density and distances between individual trees were measured in ArcView GIS (v3.2). Trees for the mating system study were selected based on their distance to nearest conspecific (*distanceNC*) with the aim to represent the range of isolation distances experienced by trees (5-350m). Paddock trees were also selected based on the average density of conspecifics (e.g. low, medium,

high). Essentially, low density trees occurred at a density of <1 tree/ha, medium density trees, 2-5 trees/ha, and high density trees, 5+ trees/ha. High density paddock trees effectively represent trees found at the same densities as in natural vegetation with the exception that no native understorey was present.

Seeds for mating system analyses were collected in two flowering seasons for each species (*E. camaldulensis* 2000 and 2002; *E. leucoxylon* 2001 and 2003). In 2002, a number of *E. camaldulensis* trees shed their seed before it could be collected so the maternal trees sampled in this year differed from those sampled in 2000. Essentially the same *E. leucoxylon* maternal trees were sampled in both years, with the exception of some trees that failed to produce enough seed for analysis.

6.2.1.2   Seedlings

Capsules were collected from maternal trees and seeds were extracted from the capsules as described in Chapter 5. Multiple capsules (~10 capsules) were collected from different positions in the canopy (~10 different positions) resulting in approximately 100 capsules per tree. For each tree I pooled the capsule contents from groups of capsules collected at each canopy position (referred to as seedlots), which were then germinated as described in Chapter 5. I randomly selected one seedling from each seedlot for genetic analysis such that ~10 seedlings (each from a different canopy position) were sampled per individual tree per flowering season. I deliberately avoided sampling multiple seedlings from the same seedlot as closely-located flowers are likely to have received pollen from the same source and this may bias outcrossing and correlated paternity analyses. Sample sizes were necessarily kept small due to the high cost of microsatellite genotyping, however, preliminary analysis showed that at least one outcrossed individual would be detected in a sample size of 10 seedlings. Seedlings were stored at $-20°C$ prior to genetic analysis. A total of 615 *E. camaldulensis* seedlings (2000 $n= 273$, 2002 $n=342$) and 423 *E. leucoxylon* seedlings (2001 $n=208$, 2003 $n=215$) were analysed for genetic diversity and outcrossing rates (details of the number of seedlings sampled per adult are provided in Appendix 3).

6.2.2 Genetic Analyses

6.2.2.1 DNA extraction and microsatellite analysis

Genomic DNA was extracted from adult leaf material and seedlings using either a CTAB method or the MasterPure™ Plant Leaf DNA Purification Kit as described in Chapter 2. Adults and seedlings were genotyped at seven (*E. camaldulensis*) or eight (*E. leucoxylon*) microsatellite loci. Reaction conditions, PCR cycling and fluorescence-based genotyping conditions were the same as described in Chapter 2.

6.2.2.2 Genetic Data Analyses

Population genetic parameters (e.g. number of alleles (N_a), expected heterozygosity (H_e), observed heterozygosity (H_o) and Wright's inbreeding coefficient (F_{is})) were estimated for adult and seedling cohorts using the program GenAlEx v6.1 (Peakall & Smouse 2005). Chi-squared tests for homogeneity of allele frequencies were carried out in Genepop on the Web (Raymond & Rousset 1995, http://genepop.curtin.edu.au). The statistical significance of chi-squared tests was assessed using Fisher's exact test with 10000 randomisations.

Individual and population multi-locus outcrossing rates (t_m) and other mating system parameters (correlation of paternity (r_p), biparental inbreeding (t_m-t_s)) were estimated in the program MLTR (Ritland 1990; Ritland 2002). In addition, the number of effective mating partners ("idealised" males, all contributing with equal probability and mating panmicticly) that contribute to the observed correlation of paternity can be estimated from the inverse of r_p (Ritland 2002). In the mating system analyses, pollen and ovule frequencies were constrained to be equal. To ensure robust estimates of adult allele frequencies in the mating system analysis, allele frequencies were calculated from the entire pool of maternal parents for each species, though the actual female trees sampled for mating system estimation differed between years. Parameter estimates were bootstrapped 1000 times to determine the standard error of each parameter. In the majority of mating system analyses the significance of estimates were assessed by resampling families, however, in the test for mating system parameters versus canopy position, I resampled individuals within families as I was interested in the likelihood of outcrossing dependent on canopy position and not family.

6.2.3 Statistical analyses

I performed bivariate correlations of mating system parameters with tree size, tree isolation, seed and capsule characteristics and germination data (data presented in Chapter 5). As there was no significant difference in mating system parameters between the two seedling cohorts of each species (Table 6.2), correlations were performed on mating system estimates from pooled data. When data were normally distributed, Pearson's correlation coefficient (r) and the significance (P) are reported. If data failed normality tests and transforms were unable to normalise the data, non-parametric correlations were performed. In this case, Spearman's rank coefficient (ρ) and the significance (P) are reported. All correlations were performed in SPSS v12.

6.3 Results

6.3.1 Genetic diversity estimates

In both eucalypt species, adult paddock trees contained relatively high levels of genetic diversity. An average of 7.7 alleles were detected in adult *E. camaldulensis* trees (surveyed at seven microsatellite loci) and an average of 9.8 alleles were detected in *E. leucoxylon* adult trees (surveyed at eight loci) (Table 6.1a,b). In adult trees, observed heterozygosity was extremely high (H_o=0.81-0.86) and in both species, observed heterozygosity was greater than expected heterozygosity (H_e=0.72-0.77). This excess of heterozygotes detected in the adult populations is reflected in the moderately strong negative value of the inbreeding coefficient (F_{is}) for both species (F_{is}=-0.13-0.14). Under conditions of no selection and random mating, F_{is} should be close to zero. In this case, an excess of heterozygotes in adult populations may reflect strong, lifetime selection against selfed individuals.

In both species, cohorts of seedlings contained a greater number of alleles than found in the adult trees (Table 6.1a,b) and this was especially the case for *E. leucoxylon* seedlings (Max. number of alleles in adults = 15, Max. number of alleles in seedlings = 21). On a coarse scale, the greater number of alleles found in seedling cohorts than contained in the adult populations indicates that at least a proportion of offspring are derived from outcrossing events. However, in both species, observed heterozygosity was lower than expected heterozygosity in seedling cohorts, which also tends to indicate that selfing is prevalent in these populations. Again, the excess of homozygotes is

reflected in the strongly positive value of F_{is} (0.17-0.20) for the seedling cohorts, indicating inbreeding for both species. Overall, both seedling cohorts of both species showed significantly higher levels of homozygosity and inbreeding than the adult populations.

Notably, genetic diversity estimates (N_a, H_o, H_e, F_{is}) were remarkably similar between the two cohorts of seedlings for both *E. camaldulensis* and *E. leucoxylon* (Table 6.1a,b), with only minimal variation in the total number of alleles detected in the seedling cohorts between years. While individuals may potentially vary in their mating patterns from year to year, this result suggests that the net population genetic outcome may essentially be the same.

To determine whether the seedling pool was genetically representative of adult trees, I performed chi-squared tests for homogeneity of allele frequencies between adults and seedling cohorts (allele frequencies for adults and seedling cohorts of each species are contained in Appendix 2). There was no significant difference between *E. camaldulensis* adults and either cohort of seedlings (2000 cohort, χ^2=11.53, df=12, P=0.64; 2002 cohort, χ^2=5.29, df=12, P=0.98). However, there was significant heterogeneity of allele frequencies between the 2000 and 2002 cohorts of *E. camaldulensis* seedlings (χ^2=∞, df=12, P=<0.0001), but the two cohorts were only weakly genetically differentiated (R_{st} = 0.001). Similarly, *E. leucoxylon* adult allele frequencies were not significantly different to seedling cohorts (2001 cohort, χ^2=5.39, df=14, P=0.99; 2003 cohort χ^2=5.48, df=14, P= 0.99), but there was significant heterogeneity of allele frequencies between the two cohorts of *E. leucoxylon* seedlings (χ^2=264.5, df=14, P=<0.0001). The two cohorts were only weakly differentiated (R_{st} = 0.004). Again, these results suggest that individual mating patterns may vary from year to year but on a gross level, seedling cohorts are genetically representative of the adult populations.

Table 6.1: Genetic diversity estimates (± S. E.) for adults and two cohorts of seedlings of *E. camaldulensis* and *E. leucoxylon* paddock trees.

N = number of individuals surveyed; N_a = mean number of alleles across loci; N_a (Range) = range of number of alleles per locus; H_o = mean observed heterozygosity; H_e = mean expected heterozygosity; F_{is} = Wright's inbreeding coefficient.

a. *E. camaldulensis*

Cohort	N	N_a	N_a (Range)	H_o	H_e	F_{is}
Adults	48	7.7 ± 0.9	5-11	0.81 ± 0.04	0.72 ± 0.03	-0.13 ± 0.03
Seedlings 2000	273	8.4 ± 1.2	5-13	0.59 ± 0.04	0.71 ± 0.03	0.17 ± 0.04
Seedlings 2002	342	8.4 ± 0.7	7-11	0.59 ± 0.04	0.72 ± 0.03	0.18 ± 0.03

b. *E. leucoxylon*

Cohort	N	N_a	N_a (Range)	H_o	H_e	F_{is}
Adults	28	9.8 ± 1.1	6-15	0.86 ± 0.03	0.77 ± 0.04	-0.14 ± 0.06
Seedlings 2001	208	13.4 ± 1.7	7-21	0.62 ± 0.05	0.77 ± 0.05	0.20 ± 0.03
Seedlings 2003	215	12.5 ± 1.5	7-19	0.63 ± 0.04	0.76 ± 0.05	0.17 ± 0.03

6.3.2   Population-level mating system estimates

Previous estimates of outcrossing rates for both *E. camaldulensis* and *E. leucoxylon* indicate that each species is predominantly outcrossing, but is capable of selfing (*E. camaldulensis* t_m = 0.75 (Moncur *et al.* 1995); *E. leucoxylon* t_m=0.83 (Ellis & Sedgley 1993). Both of these estimates are for trees found in high density populations. For example, in natural populations in the MLR, *E. camaldulensis* and *E. leucoxylon* typically occur at densities of around 100 trees/ha. For paddock trees in this study, average conspecific tree density is approximately 1 tree/ha, representing an enormous decline in the number of potential mating partners for these trees. Nevertheless, both populations of paddock trees maintained high outcrossing rates in the two years each species was surveyed. Across the population of *E. camaldulensis* scattered trees at Tungkillo, outcrossing rate in both seedling cohorts was high (68-79%, Table 6.2a) and when averaged across the two years, was close to the value of 75% found by Moncur (1995). Similarly, *E. leucoxylon* paddock trees also maintained high outcrossing rates (~82%), again remarkably similar to the estimate of 83% estimated by Ellis & Sedgley (1993) for trees found in high density populations. Despite the enormous decline in tree density experienced by these trees, population outcrossing rates do not appear to be significantly different to those for trees found in natural populations.

As suggested by the positive value of the inbreeding coefficient (F_{is}) in Section 6.3.1, both species experienced a degree of inbreeding due to self-pollination. However, across each population, paddock trees also experienced significant biparental inbreeding (the amount of inbreeding due to consanguineous matings) (13-18%, Table 6.2). This result is reasonably high considering that the majority of trees are located distant to conspecifics and there was little genetic structure detected in the populations overall (Figure 4.5), but may be driven by the small numbers of trees that were located close to genetically-related near neighbours.

The correlation of paternity parameter measures the probability of any two outcrossed offspring having the same paternal parent and the number of effective mating partners that contribute to the observed correlation of paternity can be estimated from the inverse of r_p (Ritland 2002). Levels of correlated paternity were reasonably low for *E. camaldulensis* paddock trees (17%) but slightly higher for *E. leucoxylon* trees (26%).

Interestingly, a greater number of "effective" males contributed to the seed crop of *E. camaldulensis* (predominantly insect-pollinated) trees, than *E. leucoxylon* (predominantly bird-pollinated) trees. In particular, in 2000 an average of ten effective males contributed to the seed crop of *E. camaldulensis* individuals, almost three times the number observed to contribute to the seed crop of *E. leucoxylon* individuals (Table 6.2).

Mating system parameters were not statistically significantly different between years (95% CI's overlap) for each tree species. Overall though, there was a greater difference in mating system parameter estimates of *E. camaldulensis* paddock trees between the two years surveyed. In 2000, *E. camaldulensis* paddock trees had a higher rate of selfing, but of the outcrossed seedlings, these were fathered by a greater diversity of mating partners. In contrast, in 2002 *E. camaldulensis* had a high outcrossing rate but fewer individuals fathered outcrossed seedlings. *Eucalyptus leucoxylon* population mating system estimates were very consistent between years.

6.3.3   Family-level mating system estimates

Mating system results for individual trees of each species are contained in Appendix 3.

Multi-locus outcrossing rates for individual *E. camaldulensis* paddock trees ranged from 7% to 100%, with the majority of trees having an outcrossing rate of 75% or higher (Figure 6.1a). Similarly, outcrossing rates of *E. leucoxylon* paddock trees varied greatly between individuals, from 29% to 100%, though *E. leucoxylon* tended to have a greater proportion of trees with high outcrossing rates than *E. camaldulensis* (50% of *E. leucoxylon* trees sampled had an outcrossing rate of 90% or higher) (Figure 6.1b). Biparental inbreeding rates (t_m-t_s) were low to moderate for the majority of individuals in each species, though some individuals had very high rates of biparental inbreeding (*E. camaldulensis,* maximum = 0.54; *E. leucoxylon,* maximum = 0.61). Correlated paternity estimates were also low to moderate for the majority of trees of both species. While *E. leucoxylon* trees tended to have a higher proportion of individuals with high correlated paternity, *E. camaldulensis* had two trees with extremely high levels of correlated paternity (80% and 90%), indicating that these trees may only be mating with one other individual.

Table 6.2: Mating system parameters of *E. camaldulensis* and *E. leucoxylon* paddock trees, from each of two seasons and from pooled data where the two seasons were analysed together.

a. *E. camaldulensis*

Cohort	Multilocus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)	No. effective males ($1/r_p$)
Seedlings 2000	0.68 ± 0.06	0.57 ± 0.05	0.11 ± 0.02	0.11 ± 0.05	9.5
Seedlings 2002	0.79 ± 0.04	0.64 ± 0.03	0.14 ± 0.02	0.22 ± 0.05	4.5
All Data	0.74 ± 0.03	0.61 ± 0.03	0.13 ± 0.01	0.17 ± 0.03	5.7

b. *E. leucoxylon*

Cohort	Multilocus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)	No. effective males ($1/r_p$)
Seedlings 2001	0.81 ± 0.05	0.61 ± 0.05	0.20 ± 0.02	0.30 ± 0.05	3.3
Seedlings 2003	0.82 ± 0.05	0.66 ± 0.06	0.16 ± 0.02	0.27 ± 0.07	3.8
All Data	0.82 ± 0.04	0.64 ± 0.04	0.18 ± 0.02	0.26 ± 0.06	3.8

a. *E. camaldulensis*
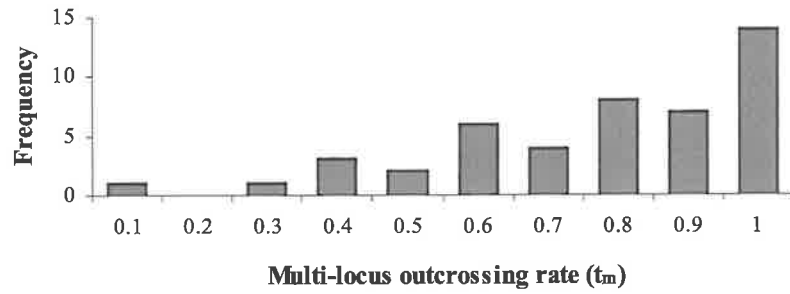
b. *E. leucoxylon*
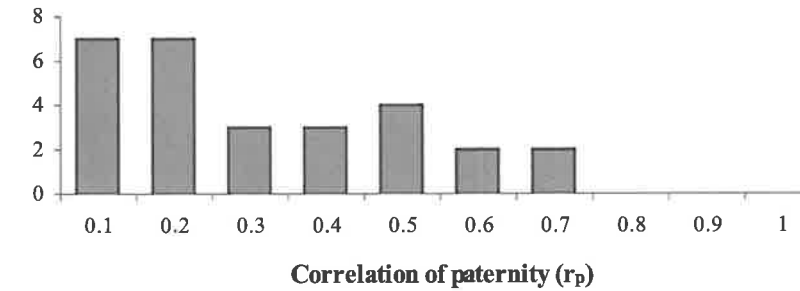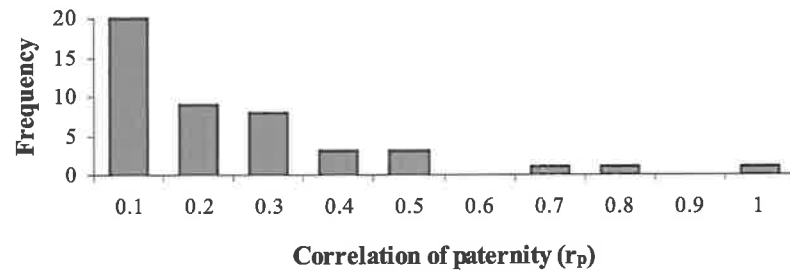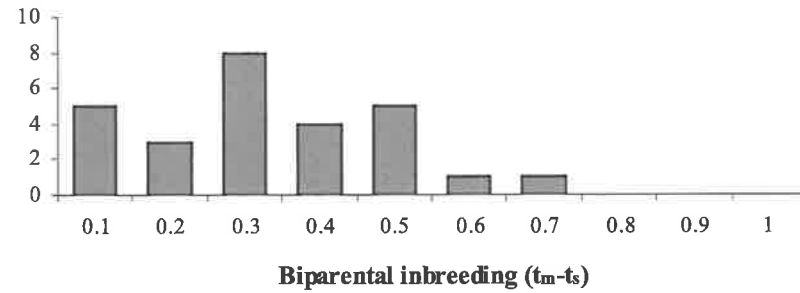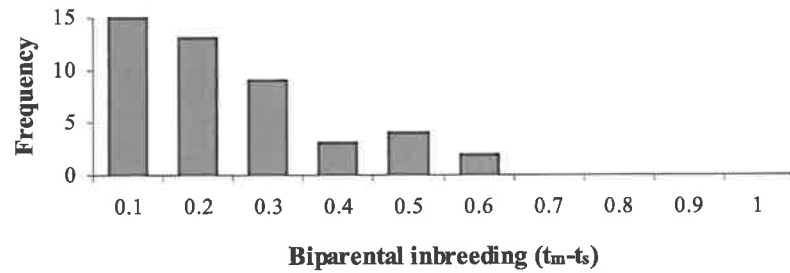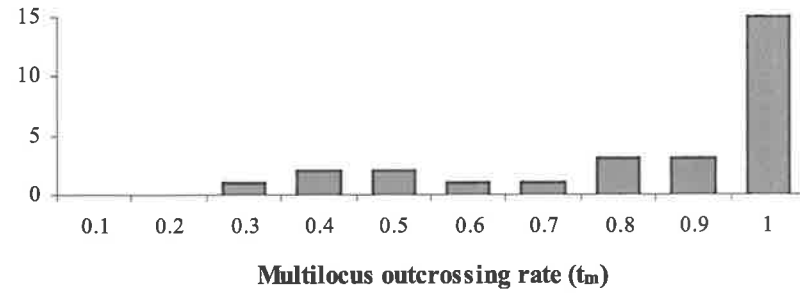
Figure 6.1: Frequency histograms of mating system parameters of individual *E. camaldulensis* (*n*=46) and *E. leucoxylon* (*n*=28) paddock trees.

6.3.4   Year to year variation in mating system estimates

All *E. camaldulensis* paddock trees that were sampled in both 2000 and 2002 showed variation in mating system parameter estimates between the two years (Figure 6.2a). Almost equal numbers of individuals had higher outcrossing rates as those that experienced a decline in outcrossing rates from 2000 to 2002. Two individuals experienced quite a large increase in outcrossing rate between the two years (Ec3287 and Ec3315), while in the majority of other individuals the changes were in the order of a 10% increase or decrease. Interestingly though, increased outcrossing rates was most frequently associated with increases in levels of biparental inbreeding.

In contrast to *E. camaldulensis*, there was very little variation in outcrossing rates of *E. leucoxylon* trees sampled in 2001 and 2003 (Figure 6.2b). There were two trees (El5608 and El5623) that had a large decline in outcrossing rate over the two years but these trees also experienced a decline in the correlation of paternity of the seed crop. Thus, while they received less outcrossed pollen, they received pollen from a greater number of mating partners. While outcrossing rates of individual *E. leucoxylon* trees were very consistent between the two years, more subtle changes in mating patterns occurred. There was moderate variation in the levels of biparental inbreeding between years for these trees, and for some trees the level of correlated paternity varied dramatically between years. The majority of trees experienced lower rates of correlated paternity in 2003 than in 2001, indicating that trees were mating with a greater number of individuals in 2003. These subtle changes in mating system parameters may reflect variation in flowering patterns (e.g. timing, fecundity) between years that affect the availability of potential mates.

a. *E. camaldulensis* (Difference 2000 & 2002 cohorts)
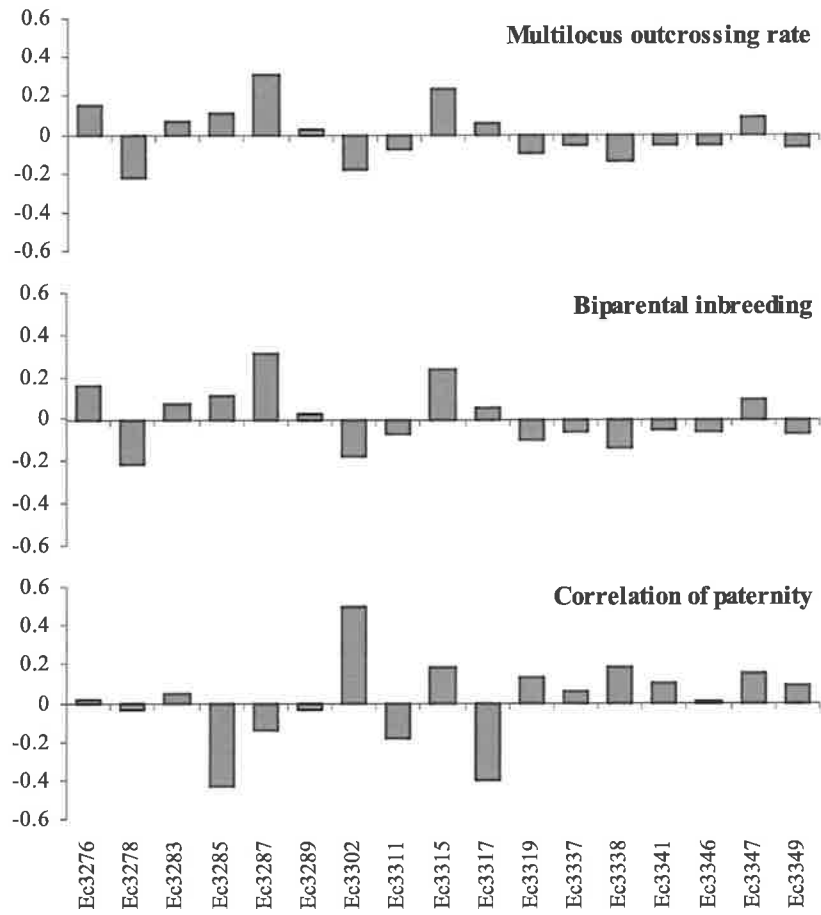
b. *E. leucoxylon* (Difference 2001 & 2003 cohorts)
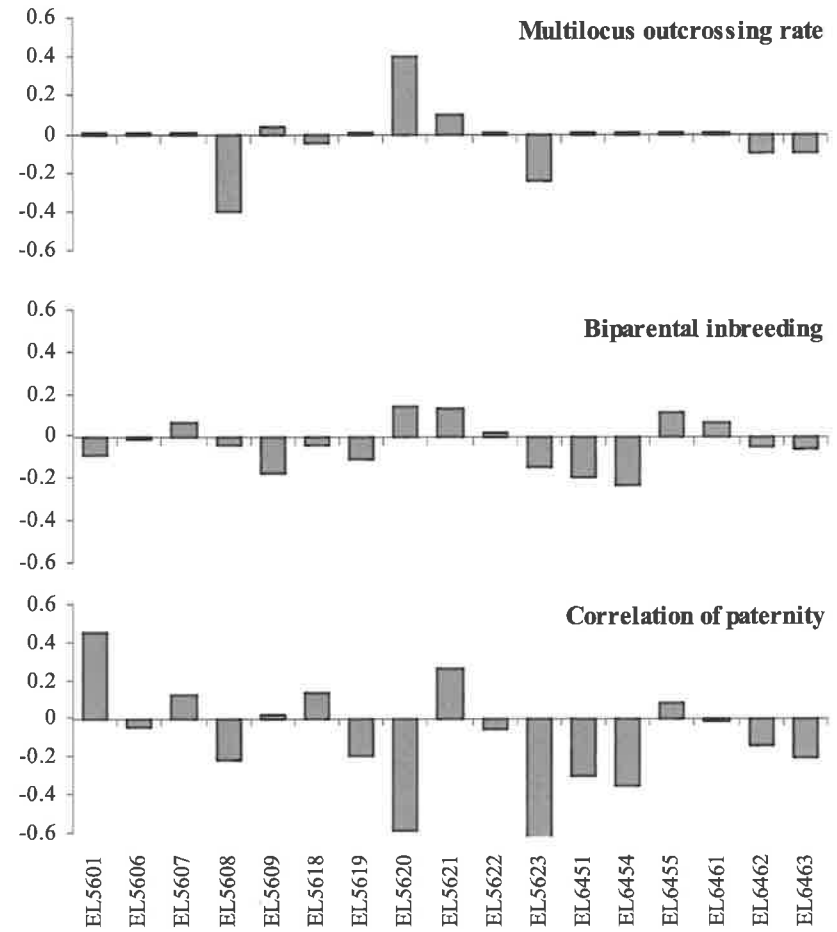
Figure 6.2: Difference in mating system parameters between two cohorts of seedlings of *E. camaldulensis* and *E. leucoxylon* trees

6.3.5   Variation in mating system parameters with canopy position

In Chapter 5, I found that capsules collected from the upper canopy of *E. camaldulensis* and *E. leucoxylon* trees had lower germination rates than seeds collected from the mid and lower canopy.   This would have suggested that flowers in the upper canopy receive fewer outcrossed pollen but as Table 6.3a shows, there was a trend for outcrossing rate to be high in seedlings collected from the upper canopy in *E. camaldulensis* trees and decline lower in the canopy (though this was not statistically significant).   Unfortunately few seeds were collected from the upper canopy of *E. leucoxylon* trees but there does not appear to be any trend in outcrossing rate with canopy position for these trees (Table 6.3b).   Interestingly, seeds collected from the upper canopy of *E. camaldulensis* trees had high levels of correlated paternity.   This may suggest that outcrossed pollen loads are deposited on upper canopy flowers when pollinators enter the canopy.   The amount of outcrossed pollen carried by pollinators may decline as they move downwards through the canopy (or may be diluted by selfed pollen) leading to lower outcrossing rates and lower correlated paternity in lower canopy fruits.   However, the opposite trend appears to occur in *E. leucoxylon* trees, with higher levels of correlated paternity in the lower canopy fruits.

Table 6.3: Mating system parameters ($\pm$ SE) for seeds collected from the lower, mid and upper canopy of *E. camaldulensis* and *E. leucoxylon* trees.

n = number of seedlings analysed

a. *E. camaldulensis*

Canopy position	n	Multilocus outcrossing rate (t_m)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)
Lower	258	0.73 ± 0.02	0.11 ± 0.02	0.12 ± 0.05
Mid	237	0.78 ± 0.03	0.17 ± 0.02	0.18 ± 0.05
Upper	50	0.82 ± 0.05	0.17 ± 0.05	0.29 ± 0.02

b. *E. leucoxylon*

Canopy position	n	Multilocus outcrossing rate (t_m)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)
Lower	252	0.83 ± 0.05	0.19 ± 0.02	0.32 ± 0.08
Mid	143	0.81 ± 0.06	0.16 ± 0.03	0.19 ± 0.07
Upper	3	1.0 ± 0.36	0.42 ± 0.16	0.99 ± 0.18

6.3.6 Tree demography versus mating system parameters

6.3.6.1 Tree size

In Chapter 5, I found that tree size was negatively correlated with germination rate in *E. camaldulensis* and *E. leucoxylon* paddock trees, potentially indicating higher levels of selfing for larger trees. However, there was very little relationship between tree size and mating system parameters for either *E. camaldulensis* or *E. leucoxylon* trees (Table 6.4a,b).

6.3.6.2 Tree isolation

In Chapter 5, I found that the degree of tree isolation (distance to nearest neighbour and nearest conspecific) was negatively correlated with germination rate in *E. leucoxylon* trees suggesting that more isolated trees receive fewer outcrossed pollen. In contrast, there was no relationship between tree isolation and germination rate in *E. camaldulensis* trees suggesting that pollinator visits are adequate over all distances measured in this study. As Table 6.4(a,b) shows, there was no significant correlation between isolation distance and outcrossing rate (or any of the mating system parameters) for either species.

Table 6.4: Spearman's rank correlation coefficients (ρ) of tree size and tree distance factors with mating system parameters.

Significance of correlations (*P*) in parentheses. *DistanceNN* = Distance to nearest neighbour (any eucalypt species); *DistanceNC* = Distance to nearest conspecific.

a. *E. camaldulensis*

Factor	Multilocus Outcrossing Rate (t_m)	Single-locus Outcrossing Rate (t_s)	Biparental Inbreeding (t_m-t_s)	Correlation of Paternity (r_p)
Tree Size	-0.09 (0.62)	-0.04 (0.83)	-0.02 (0.92)	-0.16 (0.40)
DistanceNN	-0.15 (0.32)	-0.04 (0.79)	-0.25 (0.09)	-0.12 (0.43)
DistanceNC	-0.12 (0.45)	-0.02 (0.16)	-0.21 (0.16)	-0.14 (0.36)

b. *E. leucoxylon*

Factor	Multilocus Outcrossing Rate (t_m)	Single-locus Outcrossing Rate (t_s)	Biparental Inbreeding (t_m-t_s)	Correlation of Paternity (r_p)
Tree Size	0.10 (0.64)	0.21 (0.31)	-0.13 (0.52)	-0.23 (0.26)
DistanceNN	0.10 (0.63)	0.19 (0.36)	-0.13 (0.53)	-0.31 (0.13)
DistanceNC	-0.17 (0.42)	-0.06 (0.79)	-0.27 (0.19)	-0.25 (0.21)

Indeed, individuals of both species showed large variation in all mating system parameters over the range of distances sampled (Figure 6.3). For *E. camaldulensis*, the multilocus outcrossing rate tended to decrease with increasing tree isolation, though a number of trees located up to 200m distant from conspecifics still had an outcrossing rate of 100% and even one of the most distantly located trees (>300m) had an outcrossing rate of over 60%. Though there is undoubtedly a lack of replication of trees at large distances from the nearest conspecific (i.e. >200m), potentially there is a trend for the *variation* in outcrossing rate to increase with increasing tree isolation. For example, six trees found at approximately 200m from the nearest conspecific had outcrossing rates varying from 7 to 100%.

In *E. camaldulensis* the rate of biparental inbreeding declined with increasing distance to nearest conspecific, which may be expected as trees at greater distances have fewer near neighbours than trees located close to other conspecifics. There was very little relationship between the level of correlated paternity and the distance to nearest conspecific, with the majority of trees having low to moderate levels of correlated paternity. Interestingly, the high values of correlated paternity occur at the two extremes of tree isolation, i.e. trees in close proximity to other individuals (<50m) and trees extremely distant to other individuals (>300m).

As for *E. camaldulensis,* there were no significant correlations between tree isolation and any mating system parameters in *E. leucoxylon* (Table 6.4b). As Figure 6.3b shows, outcrossing rate was generally high in *E. leucoxylon* trees located close to conspecifics. However, one tree located 380m from another individual had an outcrossing rate of 100%. The level of biparental inbreeding was quite variable for trees <50m from the nearest conspecific (Figure 6.3b) but seemed to decline over greater distances. Similarly the rate of correlated paternity was variable for trees <50m to the nearest conspecific, but overall there was a trend for the correlation of paternity to decline as the distance between individuals increased. That is, isolated trees are potentially mating with a greater diversity of individuals than trees in close proximity to other individuals.

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a. *E. camaldulensis*   b. *E. leucoxylon*
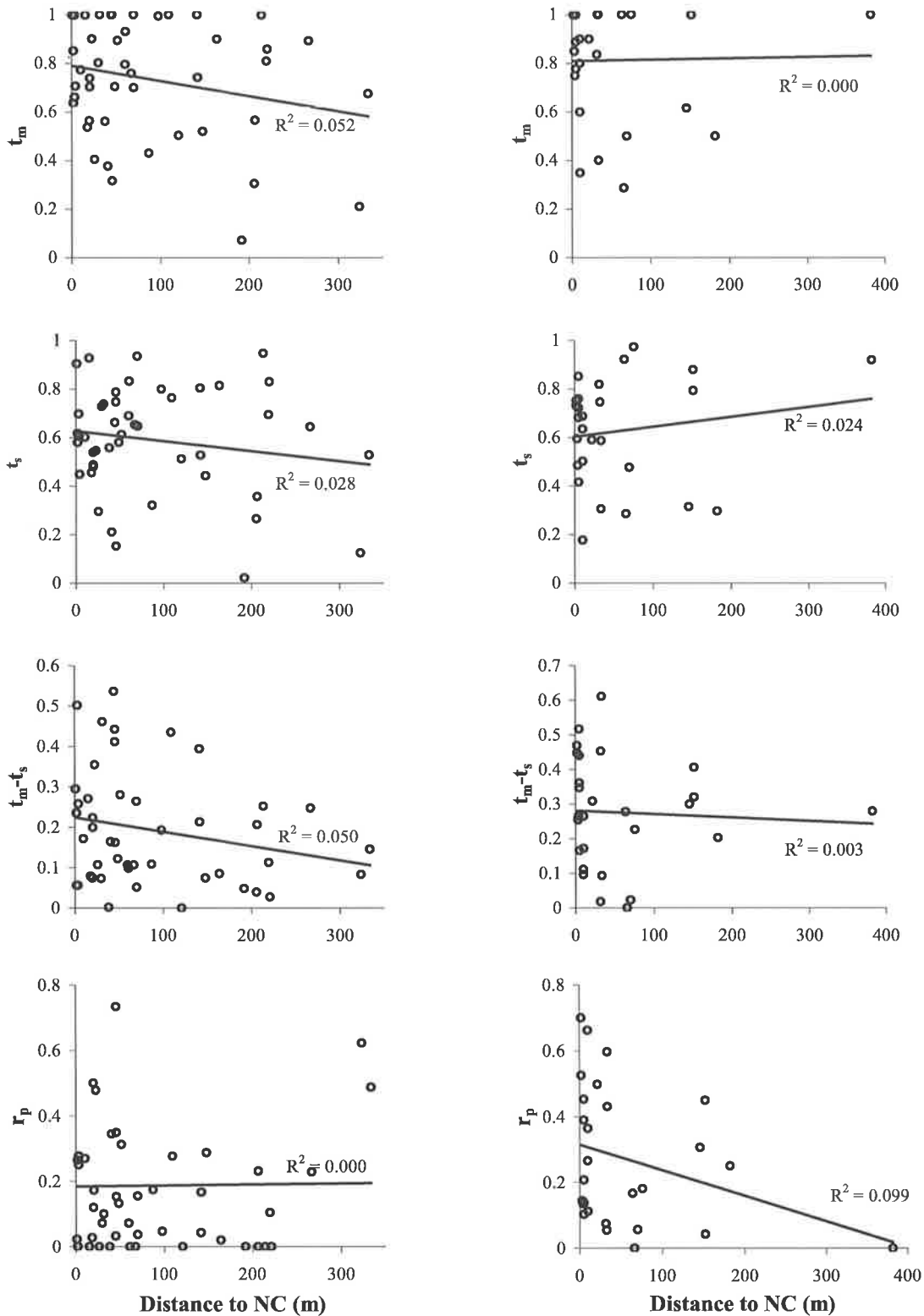
Figure 6.3: Scatter plots of mating system parameters against distance to nearest conspecific for *E. camaldulensis* and *E. leucoxylon* trees.

t_m = Multilocus outcrossing rate; t_s = Single locus outcrossing rate; t_m-t_s = Biparental inbreeding; r_p = Correlation of paternity

6.3.6.3   *Mating system parameters of trees in natural vegetation versus paddock trees*

In chapter 5 I found that trees in intact natural vegetation produced a greater weight of capsule contents to capsule weight than isolated trees but germination rate did not differ significantly between trees in the two vegetation types. Similarly, mating system parameters were not significantly different between trees in natural vegetation and isolated trees of either species, despite the enormous reduction in tree density for isolated trees (Table 6.5). However, in both species, there was a trend for isolated trees to have lower outcrossing rates and a lower rate of biparental inbreeding than trees in natural vegetation. This lower rate of biparental inbreeding may be expected for isolated trees, as few trees are located close to other individuals. Interestingly, patterns of correlated paternity differ between *E. camaldulensis* and *E. leucoxylon* trees in the different vegetation types. In *E. camaldulensis*, levels of correlated paternity were very similar between the two vegetation types and suggest that trees are mating with ~5 effective males. However, correlated paternity was greater in isolated *E. leucoxylon* trees than trees in natural vegetation. Isolated *E. leucoxylon* trees are, on average, mating with four effective males, while trees in natural vegetation are mating with ~6 males.

Table 6.5: Estimates of mating system parameters (± S.E.) for trees found in natural vegetation or as isolated trees.

n = number of progeny sampled.

Vegetation type	n	Multilocus outcrossing rate (t_m)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)	No. effective males ($1/r_p$)
E. camaldulensis					
Natural	52	0.78 ± 0.13	0.25 ± 0.13	0.22 ± 0.05	4.5
Isolated trees	563	0.74 ± 0.04	0.19 ± 0.02	0.19 ± 0.05	5.3
E. leucoxylon					
Natural	61	0.87 ± 0.21	0.25 ± 0.08	0.18 ± 0.06	5.5
Isolated trees	362	0.81 ± 0.05	0.17 ± 0.02	0.26 ± 0.07	3.8

6.3.6.4   Local conspecific density versus mating system parameters

To assess the impact of local tree density (an indication of the number of available mating partners) on the mating system of isolated trees, I compared the mating systems of paddock trees found in low, medium and high-density situations. Low density trees are trees existing at densities of <1 tree/ha, medium density trees, up to 5 trees/ha and high density trees are found at densities similar to that in natural vegetation but no natural understorey is present. In both species, no significant differences in mating system parameters were detected (95% CI's overlap) but some interesting patterns emerged.

For *E. camaldulensis*, trees found in low-density situations experience a relatively large decline in outcrossing rate when compared to trees in natural vegetation (67% vs 78%, respectively) (Figure 6.4a). However, trees in medium and high density configurations do not significantly differ in outcrossing rate to trees found in natural vegetation (77-81% vs 78%), and even medium density trees tended to have a slightly higher outcrossing rate than natural vegetation trees. As could be expected, levels of biparental inbreeding were low in low density trees compared to trees in natural vegetation (15% vs 25%, respectively). Interestingly, levels of correlated paternity were low in low and medium density paddock trees, indicating that these trees are mating with a wider range of individuals than trees found in high density situations and natural vegetation. The effective number of males contributing to the observed correlation of paternity result suggests that low and medium density trees are mating with 5-7 individuals while high density trees and trees in natural vegetation are mating with 3-4 individuals (Figure 6.5).

For *E. leucoxylon* trees, outcrossing rate did not vary as substantially between low density paddock trees and trees in natural vegetation (80% vs 87% respectively, Figure 6.4b) as it did for *E. camaldulensis* trees. The greatest difference in outcrossing rates was between medium density and high density trees (78% and 89%, respectively). Again, levels of biparental inbreeding were lower in paddock trees than trees in natural vegetation. In contrast to *E. camaldulensis*, low and medium density paddock trees had higher levels of correlated paternity than trees in natural vegetation, suggesting that these trees mate with fewer individuals. Indeed, the estimated number of effective males was 3-4 for low and medium density paddock trees, compared to 6-9 males for

high density paddock trees and trees in natural vegetation (Figure 6.5). These different patterns of correlated paternity may potentially reflect differences in the way insect and bird pollinators forage in high density plant arrays.

Furthermore, it is interesting to note that estimates of the number of effective males are very similar between *E. camaldulensis* and *E. leucoxylon* found in natural vegetation and that the number of effective males contributing to the seed crop declines with increasing tree isolation in the bird-pollinated species, *E. leucoxylon,* and increases in the insect-pollinated species, *E. camaldulensis*. Again this suggests that pollinator responses to tree isolation are very different.

6.3.7   Reproductive characteristics versus mating system parameters

In both *E. camaldulensis* and *E. leucoxylon* trees, there was a positive association between outcrossing rate and the average weight of capsule contents, and a significant negative correlation between weight of capsule contents and the level of correlated paternity (Table 6.6). This result is interesting as it suggests that trees that mated with a greater number of individuals produced heavier (or more seeds) – perhaps this indicates maternal resource allocation towards more genetically divergent seeds. In contrast though, in *E. camaldulensis* the germination rate was significantly *positively* correlated with correlated paternity, suggesting that trees with fewer mating partners produced more viable seed. There was very little association between germination rate and outcrossing rate in *E. camaldulensis*.

In *E. leucoxylon*, germination rate was very strongly associated with both the multilocus and single locus outcrossing rate ($P<0.01$). Since there was not such a strong positive correlation between the weight of capsule contents and outcrossing rate, this suggests that there may be strong selection against selfed progeny at the germination stage.

a. *E. camaldulensis*
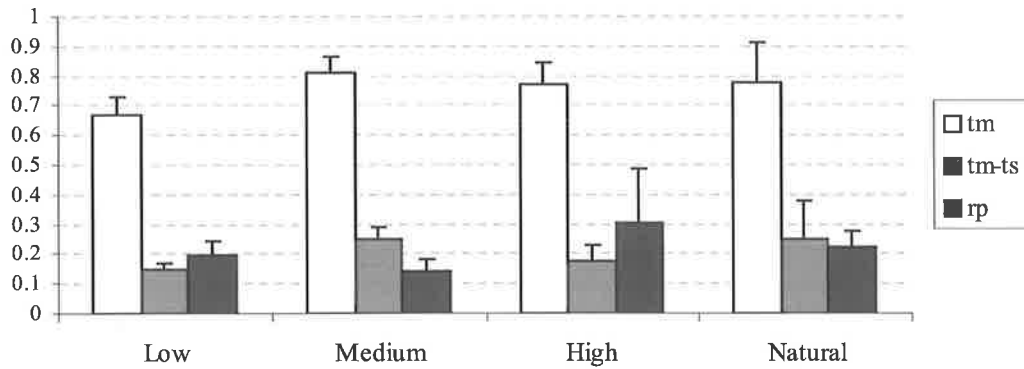
b. *E. leucoxylon*
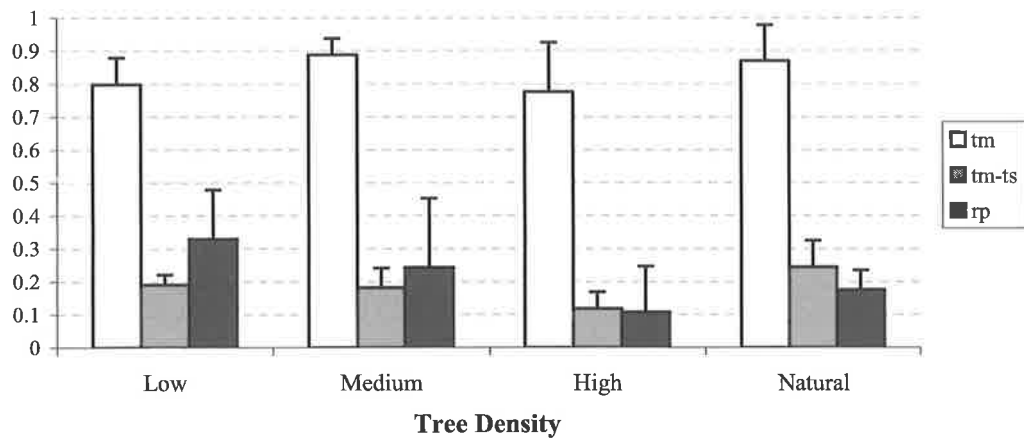
Figure 6.4: Mating system parameters of trees found in natural vegetation and isolated trees at low, medium and high density.

t_m = Multilocus outcrossing rate; t_m-t_s = biparental inbreeding; r_p = correlation of paternity. Error bars are ± S.E.
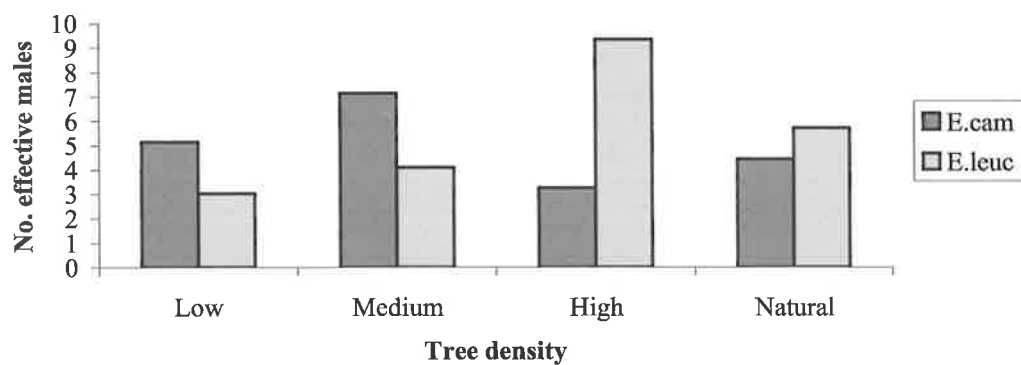
Figure 6.5: Estimated number of effective males for *E. camaldulensis* and *E. leucoxylon* trees in natural vegetation and isolated trees at low, medium and high density.

Table 6.6: Correlation coefficients (r) and significance (*P*) of averaged capsule characteristics, germination rate and mating system parameters for *E. camaldulensis* and *E. leucoxylon* trees.

Parametric Pearson's correlation coefficients (r) are reported for *E. camaldulensis* and *E. leucoxylon* variables, with the exception of multilocus outcrossing rate in *E. leucoxylon* (non-parametric Spearman's rank correlation coefficient (ρ)). Significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$

a. *E. camaldulensis*

Reproductive measure	Multilocus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Biparental inbreeding (t_m-t_s)	Correlation of paternity (r_p)
Weight of capsule contents	0.24 (0.18)	0.34 (0.06)	-0.02 (0.93)	-0.45 (0.01) **
Weight of empty capsule	-0.00 (0.99)	0.04 (0.84)	-0.05 (0.78)	-0.26 (0.16)
Capsule diameter	-0.11 (0.57)	-0.05 (0.78)	-0.21 (0.26)	-0.36 (0.04) *
Ratio weight capsule contents to capsule weight	-0.29 (0.11)	0.37 (0.04) *	-0.03 (0.89)	0.19 (0.31)
Germination rate	-0.01 (0.97)	-0.02 (0.94)	0.03 (0.86)	0.37 (0.04) *

b. *E. leucoxylon*

Reproductive measure	Multilocus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Biparental Inbreeding (t_m-t_s)	Correlation of paternity (r_p)
Weight of capsule contents	0.16 (0.42)	0.35 (0.07)	-0.20 (0.32)	-0.39 (0.04) *
Weight of empty capsule	0.29 (0.14)	0.63 (0.00) **	-0.31 (0.11)	-0.25 (0.21)
Capsule diameter	0.24 (0.22)	0.60 (0.00) **	-0.27 (0.18)	-0.28 (0.15)
Ratio weight capsule contents to capsule weight	-0.09 (0.67)	-0.19 (0.34)	0.06 (0.77)	-0.32 (0.10)
Germination rate	0.47 (0.01) **	0.56 (0.00) ***	0.11 (0.59)	0.21 (0.30)

6.4 Discussion

6.4.1 Genetic diversity parameters

Patterns of genetic diversity within paddock tree populations of *E. camaldulensis* and *E. leucoxylon* closely reflect those reported for other eucalypt species in natural populations. Adult paddock trees of both *E. camaldulensis* and *E. leucoxylon* were highly heterozygous and showed an excess of heterozygotes relative to homozygotes (reflected in the negative value of F_{is}). In contrast, all seedling cohorts had a lower level of heterozygosity compared to adults and contained an excess of homozygotes (positive F_{is} values). Many other eucalypt species exhibit a similar trend – estimates of adult F_{is} range from -0.10 to –0.30 and progeny F_{is} from +0.10 to +0.20 in a range of eucalypt species (Fripp 1982; Yeh *et al.* 1983; Sampson *et al.* 1989; Sampson *et al.* 1995). A strong negative value of F_{is} for the adult cohorts of each species may suggest strong selection against homozygous individuals throughout the lifetime of the plant. This has been shown to occur in eucalypt species in long-term field trials. For example, in long-term field trials of *E. globulus* and *E. regnans*, selfed progeny had much lower survival rates than outcrossed progeny resulting in a higher proportion of outcrossed individuals being represented in plots after 4-15 years growth (Griffin & Cotterill 1988; Hardner & Potts 1995a; Hardner & Potts 1997).

Positive inbreeding values amongst progeny are not unexpected as the majority of eucalypts are capable of selfing and thus will display an excess of homozygous genotypes in progeny arrays. The F_{is} value of *E. camaldulensis* and *E. leucoxylon* paddock trees (F_{is}=0.17-0.20) is towards the upper range of estimates from other eucalypt species in natural populations. Such a high value is potentially due to increased inbreeding as a result of tree clearance in these populations. However, estimating the inbreeding coefficient from progeny arrays (i.e. from seeds with the same maternal parent) may be problematic due to a certain degree of autocorrelation within families, leading to an inflated value of F_{is}. While this is the accepted practice in conservation genetic studies it may be more appropriate to estimate F_{is} from a random sample of offspring from across the population to avoid biasing the estimate.

In both species, the offspring of isolated trees captured a significant proportion of the genetic variation present in the adult tree population. All alleles detected in the

maternal adults were detected in the seedling cohorts, as well as additional alleles not sampled in the adults. Furthermore, there was no significant difference in allele frequencies between adults and their seedling cohorts, suggesting that offspring are genetically representative of the adult population. Despite some inbreeding, heterozygosity of seedling cohorts were relatively high – observed heterozygosity of seedlings of both species was approximately 75% of the observed heterozygosity of adults. There was significant variation in allele frequencies between years for seedling cohorts of both species, suggesting some variation in mating patterns between seasons, but overall, population genetic parameters (e.g. number of alleles, observed heterozygosity etc) were remarkably similar between seedling cohorts. These features suggest that seeds collected from paddock trees at Flaxley and Tungkillo would be suitably genetically-representative of the extant population to be used in revegetation/restoration efforts.

6.4.2   Mating system

Overall, when mating system data were averaged across individuals and across years, both populations of *E. camaldulensis* and *E. leucoxylon* trees maintained relatively high effective outcrossing rates, despite the enormous changes to population structure and demography that have occurred since European settlement. Across the population, *E. camaldulensis* trees had an outcrossing rate of 74%, almost identical to a previous estimate from Moncur (1995) of 75% for trees in a natural population. *Eucalyptus camaldulensis* paddock trees had a slightly lower outcrossing rate than trees found in natural vegetation (74% c.f. 78%) in this study. Similarly, the population outcrossing rate of *E. leucoxylon* trees was high (82%) and similar to that from a previous study (83%, Ellis & Sedgley 1993). There was a slightly greater difference in outcrossing rate between *E. leucoxylon* trees in natural vegetation and paddock trees than for *E. camaldulensis* trees: *E. leucoxylon* paddock trees had an outcrossing rate of 81% while trees in natural vegetation had an outcrossing rate of 87%. Remarkably, paddock trees are maintaining outcrossing rates similar to those for trees found in natural vegetation despite the enormous reduction in tree density, from approximately 80-100 trees/ha to 1 tree/ha for paddock trees. The selfing rate has increased for paddock trees but not to the extent as that suggested for other eucalypt species found as paddock trees. For example, Butcher *et al.* (2005) found that isolated trees of *E. benthamii* were 100%

selfed, compared to selfing rates of 30-55% for *E. benthamii* in small remnant populations.

There are many aspects of a plant's mating system that may be affected by habitat fragmentation and the coincident changes in plant-pollinator interactions. Outcrossing rates of paddock trees were high but the levels of biparental inbreeding and correlation of paternity differed from trees measured in natural vegetation. In both species, population levels of biparental inbreeding were moderate (13-18%), though higher than that reported so far for other eucalypt species. In both *E. camaldulensis* and *E. leucoxylon* paddock trees, the levels of biparental inbreeding were reduced compared to trees found in natural vegetation and biparental inbreeding declined with increasing distance to nearest conspecific. This is not surprising as paddock trees have very few near neighbours – some trees may have one or two other individuals within close radius, but for the majority of trees the nearest neighbour is >50m distant. Any sub-structuring of the population (due to limited pollen or seed gene flow) is likely to have been lost through tree clearance, reducing the potential for biparental inbreeding to occur.

In plants, high levels of correlated paternity may be detected when plants are only mating with one or a few individuals. This could occur if one (or a few) trees have high male reproductive fitness and are reproductively dominant in the population, if mating is occurring with near-neighbours or if the number of potential male parents is low (e.g. Sampson 1998; Robledo-Arnuncio *et al.* 2004a). Thus, one could predict that the level of correlated paternity should be high in paddock trees compared to trees in natural vegetation primarily due to the reduction in number of potential mating partners. Across the populations of *E. camaldulensis* and *E. leucoxylon* trees, estimates of correlated paternity were moderate (r_p=0.17-0.26). Paddock trees of *E. camaldulensis* had similar levels of correlated paternity to trees in intact vegetation (19% c.f. 22%), while paddock trees of *E. leucoxylon* had higher levels of correlated paternity than trees in intact vegetation (26% c.f. 18%). Several studies have noted an increase in the level of correlated paternity for isolated trees compared to trees in continuous vegetation (Rocha & Aguilar 2001; Cascante *et al.* 2002; Fuchs *et al.* 2003). In particular, Fuchs *et al.* (2003) observed a massive increase in the level of correlated paternity of *Pachira quinata* isolated trees, 74% compared to 47% in continuous vegetation. However,

Wang *et al*. (2007) found that isolated trees of *Eurycorymbus cavaleriei* in fact had a higher number of pollen donors than trees in high density arrays.

Eucalyptus leucoxylon paddock trees experienced an increase in the level of correlated paternity despite the expectation that this may have been overcome due to the high mobility of bird pollinators in comparison to the insect-pollinated *E. camaldulensis*. *Eucalyptus leucoxylon* and *E. camaldulensis* differ slightly in their flowering strategies. *Eucalyptus leucoxylon* has moderately patchy flowering, in that individuals do not tightly overlap in flowering period and there is spot flowering throughout the year, whereas flowering in *E. camaldulensis* occurs only during a 3-4 week period. Thus, it is likely that at particular times the available number of mating partners (the *effective* tree density) for individual *E. leucoxylon* trees is much lower than the total number of trees in the landscape. The low effective density of *E. leucoxylon* trees may potentially have contributed to the higher level of correlated paternity in paddock trees, which may also be exacerbated by the patchy distribution of *E. leucoxylon* trees across the study site (Chapter 4).

In addition, it should be noted that in this study I deliberately chose to sample seeds that were widely spaced across the canopy of individual trees. Since individual pollinators tend to forage only in small sections of the canopy, sampling multiple seeds from any one small section of the canopy would potentially increase the probability of sampling seeds with the same outcrossed paternal parent (i.e. correlated paternity). In addition, only a very small number of seedlings were sampled per individual tree, considering the total potential reproductive output of trees of both species. Therefore, it is likely that the true level of correlated paternity of the total seed crop of paddock trees has been severely underestimated in this study. Furthermore, it is also likely that the total number of paternal parents contributing to the seed crop may have also been underestimated since this sampling design would be unlikely to detect the entire range of mating events that have occurred, and, for example, whether matings with particular male parents occurred at low or high frequency. In this case it would be useful to increase the number of seedlings sampled per individual tree to more thoroughly investigate within-tree patterns of paternity.

As I found with measures of flowering and fruiting in *E. camaldulensis* and *E. leucoxylon* paddock trees (Chapter 5), there was high variability in mating system estimates between individual trees. Individual outcrossing rates of *E. camaldulensis* paddock trees varied from almost complete selfing (7%) to complete outcrossing (100%) and *E. leucoxylon* trees similarly varied from 26% to 100% outcrossing. Many other authors have commented on the high variability in individual outcrossing rates for a range of *Eucalyptus* species (e.g. Brown *et al.* 1975; Peters *et al.* 1990; House & Bell 1996; McDonald *et al.* 2003; Butcher *et al.* 2005). It is hard to determine whether the variability in outcrossing rates of isolated trees has increased due to habitat clearance or whether this is a natural feature of eucalypt mating systems. While outcrossing rate varied greatly between individuals, there was less year-to-year variation in individual outcrossing rates for either species. For individuals that were sampled in both years, almost an equivalent numbers of individuals experienced an increase in outcrossing rate as experienced a decline. A few individuals experienced quite dramatic variation in outcrossing rate, but overall individual outcrossing rates were relatively consistent across years, especially for *E. leucoxylon*. In addition, at the population level, mating system estimates were more consistent between years for *E. leucoxylon* than *E. camaldulensis*. Potentially, there may be a stronger level of self-incompatibility in *E. leucoxylon* trees (for example, 50% of the trees sampled had an outcrossing rate of 90% or higher), which helps to maintain a high effective outcrossing rate across individuals and across years. In contrast, *E. camaldulensis* appears to have more flexibility in its mating system such that the realised mating system of individuals may vary in response to a range of factors (e.g. pollinator availability, availability of mating partners etc).

6.4.3   Tree isolation and conspecific density

There have been a number of studies demonstrating the association between mating system parameters and plant density. In general, for self-compatible plants, the degree of inbreeding and the level of correlated paternity has been shown to increase with decreasing plant density or with increasing plant isolation (Murawski & Hamrick 1991; Murawski *et al.* 1994; Karron *et al.* 1995; Hardner *et al.* 1996; Cascante *et al.* 2002; Fuchs *et al.* 2003).

In this study, neither *E. camaldulensis* nor *E. leucoxylon* mating system parameters were significantly associated with the degree of spatial isolation of trees (measured as the distance to nearest conspecific). There was a weak trend in both species for outcrossing rate to decline with increasing spatial isolation, but even trees isolated from other individuals by 300-400m had relatively high outcrossing rates. In addition, there was a weak trend for the level of correlated paternity to decline with increasing distance to nearest conspecific, indicating that isolated trees are indeed mating with a greater diversity of individuals than closely located trees. Both of these trends indicate that the range of isolation distances experienced by paddock trees in this study (0-400m) are within the foraging range of pollinators and effective pollen dispersal is still occurring, even in such a highly dispersed population.

Also in this study, I sampled paddock trees occurring in situations with a low, medium or high density of conspecifics to determine whether local tree density influenced mating system outcomes for each species. Again, mating system parameters were not statistically different between density treatments but some interesting trends were evident. In low density arrays (<1 tree/ha), both *E. camaldulensis* and *E. leucoxylon* trees experienced a 10-15% increase in the level of geitonogamous selfing compared to trees sampled in intact vegetation, but this was also associated with a decline in the rate of biparental inbreeding as would be expected for trees with few near neighbours. While this decline in outcrossing rate of *E. camaldulensis* and *E. leucoxylon* paddock trees was not statistically significant, other studies of a range of tree species indicate a similar effect size of a 10-15% increase in selfing (Aldrich & Hamrick 1998; Dick 2001; Cascante *et al.* 2002; Fuchs *et al.* 2003). Increasing the number of seeds sampled or the number of individuals sampled in my study may have overcome the large variability in outcrossing rates between individuals that lead to high statistical variance in mating system estimates.

Interestingly, *E. camaldulensis* and *E. leucoxylon* trees displayed slightly different patterns of correlated paternity of trees found in the different demographic categories. For *E. camaldulensis*, which is insect-pollinated, the level of correlated paternity declined as tree density declined, indicating that lower density trees were mating with a greater number of individuals than trees in high density arrays, despite an enormous

decline in the number of available mates. Indeed, the theoretical estimate of the number of effective males contributing to the observed estimates of correlated paternity (based on sample sizes of ~20 seeds per tree) was an average of 5-7 males for low to medium density trees and 3-4 males for high density paddock trees and trees in natural vegetation. This is in contrast to the trends observed for other trees with animal-dispersed pollen that occur as paddock trees (Rocha & Aguilar 2001; Cascante *et al.* 2002; Fuchs *et al.* 2003), and also in contrast to expectations based on the limited mobility and foraging behaviour of insect pollinators in comparison to bird pollinators. In contrast, in *E. leucoxylon*, the level of correlated paternity increased with declining tree density, conforming to expectations that correlated matings would increase with the declining number of available mates. In low to medium density *E. leucoxylon* paddock trees, an estimated 3-4 effective males contributed to the sampled seeds, while in high density arrays 6-9 effective males contributed.

6.4.4   Mating system parameters and tree characteristics

As I have found in previous chapters there was not a simple relationship between tree size or physical isolation and mating system parameters. In both species, there was a weak trend for outcrossing rate, as well as biparental inbreeding and the level of correlated paternity, to decline with increasing physical isolation. Thus, while isolated trees may experience an increase in geitonogamous selfing, in line with theoretical expectations (Bosch & Waser 1999; Goulson 2000; Grindeland *et al.* 2005), they are potentially also mating with a greater number of individuals.

The average size and weight of capsules and capsule contents were significantly correlated with some mating system parameters in each species. Interestingly, in *E. leucoxylon* the size and weight of capsules was significantly positively correlated with the single-locus outcrossing rate, and in both *E. camaldulensis* and *E. leucoxylon* the weight of capsule contents was significantly negatively correlated with the correlation of paternity. That is, trees that produced a greater average weight of capsule contents had mated with a greater number of individuals. Perhaps this indicates allocation of maternal resources towards outcrossed seeds, particularly seeds that are highly heterozygous as suggested by James & Kennington (1993).

Interestingly, the average germination rate of trees was strongly positively associated with outcrossing rates in *E. leucoxylon* trees, but there was no relationship in *E. camaldulensis* trees. This supports the observation that *E. leucoxylon* may indeed have display a stronger level of early-acting inbreeding depression than *E. camaldulensis* and that this may be manifest at the stage of germination (i.e. selfed seeds fail to germinate). Though, clearly, inbreeding depression is not complete in *E. leucoxylon* as a number of selfed seeds were germinated. This may indicate a type of bet-hedging strategy whereby trees that receive inadequate outcrossed pollen are still able to mature selfed seeds, whereas trees that do receive sufficient outcrossed pollen may preferentially mature outcrossed seed at the expense of selfed seed.

There is evidence that outcrossing rates may vary between the lower and upper canopy of individual eucalypt trees (Eldridge 1970; Patterson *et al.* 2001) and that this may be explained by bird pollinator behaviour (Hingston & Potts 2005), since birds most frequently enter the upper canopy (carrying outcrossed pollen) when making movements between trees. In the insect-pollinated *E. camaldulensis*, the outcrossing rate was indeed higher in seeds collected from the upper canopy and was lowest in the lower canopy. Most strikingly though, the correlation of paternity was twice as great for seeds in the upper canopy as for seeds collected from the lower canopy. While upper canopy flowers receive more outcrossed pollen, the lower canopy flowers actually receive pollen from more diverse sources. If insect pollinators also spend a greater amount of time foraging in the upper canopy than the lower canopy (as do birds) then it is more likely that pollen grains from a single source will be transferred to multiple flowers, increasing the rate of correlated paternity in upper canopy seeds. Unfortunately, I was unable to replicate the same test in *E. leucoxylon*, as it was very difficult to collect capsules from the upper canopy of these trees. However, there was very little difference in outcrossing rate between seeds collected from the lower and the mid canopy in this species.

6.5 Conclusions

The realised mating system of *E. camaldulensis* and *E. leucoxylon* paddock trees indicates relatively high levels of outcrossing, low levels of biparental inbreeding and correlated paternity, despite the enormous decline in plant density in these populations.

There was not a simple linear relationship between outcrossing rates and inter-mate distances even though optimal foraging theory would suggest that isolated trees may receive fewer pollinator visits and experience higher selfing rates due to pollinator foraging behaviour (Bosch & Waser 1999; Goulson 2000; Grindeland *et al.* 2005). The lack of a clear relationship may be due to the difference in measuring the realised mating system of these trees (as I have done in this study) compared to measuring the "true" mating system where all mating events are detectable. In plant species in which post-pollination aborted seeds are directly observable, authors have shown that perturbations to pollination systems have indeed lead to greater seed abortion rates due to increased selfing (e.g. *Picea rubens*, Rajora *et al.* 2000; *Pinus sylvestris,* Robledo-Amuncio *et al.* 2004). However, eucalypts mature many woody capsules that contain numerous small seeds making it extremely difficult to detect aborted seeds and thus measure the true selfing rate. In addition, measuring mating system parameters in post-germination seedlings may also underestimate the true selfing rate since seeds are likely to have undergone selection against selfed progeny throughout the developmental process to that stage. Ultimately though, the realised mating system measured at the seedling stage is a representation of the potential genetic contribution of plants to the next generation and is a useful starting point on which to design seed collection or revegetation strategies.

Genetic information from the progeny of *E. camaldulensis* and *E. leucoxylon* paddock trees indicate that seed collected from these trees are genetically representative of the extant adults in the population. Despite expectations that the insect-pollinated *E. camaldulensis* paddock trees may experience severe reproductive limitation due to the lower mobility of insect-pollinators, I found that even low density paddock trees maintain relatively high outcrossing rates and in fact are mating with a greater number of individuals than trees in undisturbed vegetation. This suggests that it may be suitable to collect seeds even from relatively isolated *E. camaldulensis* trees as the seed crop contains the genetic material from a diversity of trees. In contrast, low density bird-pollinated *E. leucoxylon* paddock trees, while having relatively high outcrossing rates, were mating with fewer individuals and thus it may be more suitable to collect seeds from more densely spaced trees in order to capture the maximum amount of genetic diversity of surrounding trees.

Nonetheless, it appears that inter-tree distances at each study site (up to 400m) are within the foraging range of bird and insect-pollinators as even the most isolated trees included in this study produced outcrossed offspring. Thus, while paddock trees may be spatially isolated, they are not necessarily reproductively isolated and could potentially contribute to high levels of gene flow across the landscape. Under the current grazing regime at these study sites, the female reproductive success of paddock trees is essentially zero as offspring are grazed before they can become established. In contrast, the large spatial distance of pollen gene flow suggested by these mating system results, indicates that the male reproductive success of paddock trees may be much higher. In this way paddock trees are potentially capable of providing genetic connectivity between isolated fragments of vegetation, where offspring may have more chance of survival to adulthood when protected from livestock grazing.

Chapter 7   Pollen-mediated gene flow amongst *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees

7.1   Introduction

Up until recently, the movement of pollen in plant populations has largely been invisible. Patterns of pollen dispersal have been inferred from observations of pollinator movements (e.g. Schmitt 1980; Morris 1993) or by tracking fluorescently-labelled pollen (e.g. Pacheco *et al.* 1986; Waser 1988; Campbell & Waser 1989). However, it is not appropriate to interpret such movements of pollen as gene flow, because *effective* gene flow through pollen dispersal is determined not only by the number of pollen grains received by a plant but also by genetic factors, which influence fertilisation and the development of seeds and seedlings (Waser & Price 1989; Hardner & Potts 1995a). With the advent of molecular genetic markers, such as allozymes or microsatellites, effective gene flow can be inferred through the measurement of plant mating system characteristics. Genetic information from the progeny of parental trees allows us to determine whether the progeny originated from outcrossing, selfing or apomixis. Among outcrossed progeny, it is also possible to estimate the degree of biparental inbreeding (mating with genetically related individuals), how often progeny are full sibs and the number of male parents represented in the seed crop (Ritland 2002).

While mating system analyses can provide insight into the mating patterns of individuals and populations, it provides only limited information about the individuals that contributed pollen to the seedling pool. Increasingly, conservation biologists are asking more specific questions about patterns of pollen gene flow – how far is pollen dispersed and how frequently? What characteristics determine male reproductive success? What landscape features facilitate or limit pollen dispersal? How do interactions between plants and their pollinators influence pollen dispersal? Molecular genetic markers such as microsatellites and AFLPs are sufficiently variable to discriminate between individuals with high precision, allowing investigators to accurately identify the individuals involved in successful matings using a range of parentage assignment techniques (e.g. Devlin & Ellstrand 1990; Adams & Birkes 1991; Marshall *et al.* 1998). By performing parentage-type analyses on progeny arrays we gain an understanding of the patterns of pollen gene flow, the ecological and genetic

factors that may influence them and determine the impacts of human modification of plant ecosystems.

7.1.1   Habitat fragmentation and patterns of pollen-mediated gene flow

Evidence from plant mating system analyses (reviewed in Chapter 6) and theoretical considerations suggest that patterns of pollen gene flow are likely to be reduced in fragmented habitats and may be even further restricted for isolated trees. Early studies of pollen dispersal suggested that pollen gene flow only occurred over distances of metres to tens of metres in many natural plant populations (Levin & Kerster 1974; Levin 1981; Ellstrand 2003). As such, remnant trees, often isolated from intact populations by hundreds of metres, were referred to as the "living dead", persisting by virtue of their longevity but having little or no reproductive future and contributing little to forest regeneration due to their spatial isolation (Janzen 1986). However, with the advent of molecular markers and statistical methods to measure pollen gene flow, it is now emerging that pollen dispersal occurs over much larger distances than originally thought, up to several hundred metres or even kilometres (Ellstrand *et al.* 1989; Stacy *et al.* 1996; White *et al.* 2002). Some examples of average pollen dispersal distances, estimated through parentage analysis methods, for a range of tree species are provided in Table 7.1. Thus, in contrast to the prediction that spatial isolation leads to reproductive isolation for remnant trees, this new information suggests that many pollinators are capable of traversing large distances, at least in natural forest.

Nonetheless, the impacts of habitat fragmentation and plant isolation on patterns of pollen gene flow are likely to be determined by the life history characteristics of the plant species in question. Factors that influence the frequency and distance of pollen gene flow for particular species include mating system characteristics (e.g. the presence or strength of self-incompatibility mechanisms), the degree of inbreeding depression (e.g. avoidance of selfing or biparental inbreeding through selective abortion of seeds), synchrony or asynchrony of flowering, pollination syndrome (e.g. wind vs animal-pollination); and in the case of animal-pollinated species, pollinator behaviour (e.g. specialist vs generalist pollinators, flight capabilities) (Ghazoul 2005; Aguilar *et al.* 2006).

Table 7.1: Examples of pollen dispersal distances determined by paternity analysis for a range of temperate and tropical tree species.

Species	Mean Pollen Dispersal Distance	Population type	Pollination syndrome	Mating system	Reference
Fagus sylvatica	37m	Within fragments	Wind	?	Wang 2004
Dipterocarpus tempehes	192 (1998) – 222m (1996)	Continuous population (sampled in two seasons)	Insect (giant honeybees and moths)	Partially self-incompatible	Kenta *et al.* 2004
Pinus densiflora	68m (max 325m)	Population fragment	Wind?	Partially self-incompatible	Lian *et al.* 2001
Pinus flexilis	140m	Isolated subpopulation	Wind	?	Schuster & Mitton 2000
Magnolia obovata	Outcrossed seeds = 157m Including selfed seeds = 21m	Large remnant	Insect (beetles)	Self-compatible	Isagi *et al.* 2004
Dinizia excelsa	Isolated trees = 1288m Continuous forest = 417m	Remnant trees versus continuous forest	Insect (African honeybees, native stingless bees, beetles)	Self-compatible	Dick 2001
Swietenia humilis	Fragments = 2.1km Isolated tree = >4.5km	Fragmented	Insect (bees)	Self-incompatible	White *et al.* 2002
Cercidiphyllum japonicum	129m (max = 666m)	Continuous forest	Wind	?	Sato *et al.* 2006
Gliricidia sepium	75m (max = 275m)	Continuous forest	Insect (solitary bees)	Self-incompatible	Dawson *et al.* 1997
Eucalyptus grandis	32m (max = 85m)	Seed orchard	Insect	Self-compatible	Chaix *et al.* 2003

In contrast to theoretical predictions, a number of habitat fragmentation studies have shown that the rate of gene flow into fragments is greater than that in continuous forest for a range of tree species (e.g. *Swietenia humilis,* White *et al.* 2002; *Quercus macrocarpa*, Dow & Ashley 1998a; *Acer saccharum*, Ballal *et al.* 1994; *Symphonia globulifera,* Aldrich *et al.* 1998). In wind-pollinated species (e.g. *Acer, Quercus*), a reduction in vegetation structure is thought to have lead to increased pollen dispersal (Fore 1992; Fernandez & Sork 2005), but it is less clear what aspects of pollinator behaviour leads to increased gene flow in animal-pollinated plants. White *et al.* (2002) found that there was an extensive network of gene exchange amongst even very small fragments of *Swietenia humilis*, despite the very high degree of fragmentation across their study site. For example, an isolated tree located 1.1km distant to its nearest conspecifics had the majority of its offspring fathered by individuals located in a remnant patch >4.5km distant. Similarly high levels of pollen dispersal have been recorded for *Dinizia excelsa* isolated trees (Dick 2001). In a comparison of pollen dispersal in primary forest and in paddock trees, Dick (2001) found the average distance of pollen dispersal in continuous forest was 417m, while in the remnant paddock trees the average distance was 1.3km (maximum 3.2km). Dick (2001) attributed this difference in pollen dispersal distance to the presence of African honeybees in disturbed habitat that were absent from continuous forest, and that have a greater foraging range than native bee and beetle pollinators.

7.1.2   Estimating patterns of pollen dispersal

Genetic studies of parentage have played a major role in the study of evolution and behavioural ecology (Jones & Ardren 2003) and informed many conservation decisions (Paetkau *et al.* 1995; Slate *et al.* 2000; White *et al.* 2002). There have been many statistical methods employed to assign parentage to progeny based on multilocus genetic data (e.g. Ellstrand 1984; Meagher 1986; Meagher & Thompson 1987; Devlin *et al.* 1988; Devlin & Ellstrand 1990). Currently the most popular and most refined method of parentage assignment is that based on Meagher & Thompson's (1987) work and made publicly available via the computer program *CERVUS* (Marshall *et al.* 1998). *CERVUS* is based on the categorical allocation of paternity using a maximum-likelihood approach to select the most likely parent from a pool of non-excluded parents – parentage is assigned to offspring based on the highest likelihood score between the selected parent and offspring (see Marshall *et al.* 1998 or Jones & Ardren 2003 for a

detailed description of methods). The paternity simulation option provided in *CERVUS* allows researchers to test parentage assignments with statistical confidence. Unlike other methods, *CERVUS* also provides options to control for the effects of typing error, unsampled candidate males and missing genotypes in the paternity analysis.

Due to the nature of paternity assignment tests, paternity assignment methods work best when all candidate parents in a population have been sampled and the genotypes of these individuals and progeny are known precisely. In the wild, it may be difficult to achieve these aims. Paternity-based methods are generally laborious; they require numerous, highly variable markers in order to precisely characterise individuals, and require extensive sampling of all candidate parents. Many studies of plant populations are inherently limited by not being able to sample all potential fathers in large, continuous populations (Burczyk & Koralewski 2005), especially when the potential distance of pollen dispersal (i.e. the pollination neighbourhood) is unknown.

Recently, Smouse *et al.* (2001) proposed the TWOGENER approach, which attempts to estimate the extent of pollen movement based on estimates of genetic differentiation among pools of pollen gametes effectively fertilising ovules of different mother plants (calculated as the statistic Φ_{FT}). Φ_{FT} is analogous to Wright's F_{ST} – molecular analysis of variation is used to apportion the variation in pollen pool allele frequencies to within mothers or between mothers. Thus, the TWOGENER model tests the null hypothesis of global pollen dispersal (panmixia) against the hypothesis of local pollen dispersal. The TWOGENER approach requires less sampling and genotyping compared to conventional paternity-based methods, thus it is predicted to be a more efficient way to estimate long-distance gene flow in continuous plant populations (Sork *et al.* 2002; Austerlitz *et al.* 2004). Statistical treatments have shown that the statistic Φ_{FT} is inversely related to the mean pollination distance (Austerlitz & Smouse 2001; Smouse *et al.* 2001) and the number of effective males pollinating the seed crop (Smouse *et al.* 2001). The TWOGENER approach has also been used to estimate pollen dispersal distances from a range of pollen dispersal curves (Austerlitz *et al.* 2004). The TWOGENER approach has been successfully applied to a number of study species, in order to estimate average pollination distances and to detect the effects of different landscape features or management practices on patterns of pollen gene flow (Smouse *et*

al. 2001; Sork *et al.* 2002; Dick *et al.* 2003; Degen *et al.* 2004; Robledo-Arnuncio *et al.* 2004b).

7.1.3   Pollen dispersal in *Eucalyptus*

Few studies are available to date detailing pollen dispersal distances in *Eucalyptus* species. Evidence from indirect studies and paternity analyses in seed orchards tend to indicate fairly restricted pollen dispersal distances in comparison to those for many tropical tree species (Table 7.1). For example, a recent study by Chaix *et al.* (2003), using parentage analysis of microsatellite data, indicated a mean pollen dispersal distance of 32m (max 85m) in a *Eucalyptus grandis* seed orchard. A similar figure (42m) was obtained by Adam, Griffin & Moran (cited in Sedgley & Griffin (1989)) using allozyme markers in an *E. regnans* seed orchard. Indirect evidence from eucalypt hybridisation studies indicate that the majority of pollen dispersal is local (e.g. within 100m) but that very low levels of pollen dispersal may occur over much larger distances (>500m, Potts & Reid 1988; >1.6km, Barbour *et al.* 2005).

These estimates of pollen dispersal distances for *Eucalyptus* come from eucalypt populations found in natural density or artificially high density populations (seed orchards). To date there has been no study of pollen dispersal patterns of eucalypt species in fragmented habitats, even though a great proportion of forest and woodland eucalypt species throughout Australia have been impacted upon by habitat fragmentation and clearance. A number of tropical tree species have shown to be resilient to the impacts of fragmentation (e.g. Dick 2001; White *et al.* 2002), potentially because they occur at low densities in natural forest (especially gap-colonising species) and therefore may be pre-adapted to maintaining gene flow over very large areas. However, most eucalypt species typically occur at high density in natural populations (though their distribution may be patchy due to ecological conditions) and appear to have relatively restricted pollen dispersal on a local scale. Thus, based on the estimates of pollen dispersal distances, isolated eucalypts may suffer reduced gene flow in comparison to continuous populations as the distance of separation of trees is likely to be greater than the average pollination dispersal distance identified above.

7.1.4   Patterns of pollen dispersal in *E. camaldulensis* and *E. leucoxylon* paddock trees

In this chapter I use genetic data from progeny arrays to explore patterns of effective pollen dispersal in *E. camaldulensis* and *E. leucoxylon* paddock trees. I used the TWOGENER model to initially describe the overall pattern of pollen dispersal in each population of paddock trees to determine whether patterns of pollen dispersal were reflective of panmixia or whether structuring was present. From these modelling results I estimated the number of effective pollen donors and the average distance of pollen dispersal. To give a "real time" estimate of the distance of pollen dispersal and the number of mating partners, I used statistical parentage analysis to assign paternity to seeds from known maternal trees. I contrast the two methods for estimating patterns of pollen dispersal in paddock trees.

7.2   Methods

7.2.1   Sampling

A subset of *E. camaldulensis* paddock trees was selected on which to perform paternity analysis. *Eucalyptus camaldulensis* paddock trees were located within a single paddock at Tungkillo (covering an area of approximately 15 hectares). I collected genetic material from all trees in the paddock (n=25) and these were considered potential pollen donors. Seed material was collected from throughout the canopy of seven paddock trees (referred to as "maternal trees") in 2000 and 2002 and germinated as described in Chapter 5. Approximately 10 seedlings were sampled per maternal tree in each year.

For *E. leucoxylon*, paternity analysis was performed on progeny arrays for all trees included in the mating system analysis (n=28, described in Chapter 6). Seed was collected from the canopy of maternal trees in 2001 and 2003 and approximately 10 seedlings per mother tree were sampled in each year. In addition, I included all sampled *E. leucoxylon* paddock trees (n=32) as potential pollen donors in the paternity analysis. However, this meant that there were quite a large number of unsampled pollen donors at this site and this was taken into account in the paternity analysis that follows. This approach differed to that for *E. camaldulensis* due to the patchy distribution of *E. leucoxylon* paddock trees at Flaxley. At Flaxley only a very small number of paddock trees were located in an area equivalent to that used for *E. camaldulensis*. In addition,

E. leucoxylon is bird-pollinated and thus pollen dispersal is likely occurring over a very large area and not just restricted to a few trees.

Inter-tree distances of *E. camaldulensis* and *E. leucoxylon* paddock trees were calculated from aerial photographs in ArcView v3.2 as described in Chapter 4.

7.2.2   Genetic methods

Genomic DNA was extracted from leaf material collected from adult trees and from seedlings as described in Chapter 2. Both adults and seedlings were genotyped at seven (*E. camaldulensis*) or eight (*E. leucoxylon*) microsatellite loci also described in Chapter 2. The seven microsatellite loci for *E. camaldulensis* provided an exclusionary power for paternity analysis of 98.7% when one parent is known. One locus, *Eg65*, had an excess of homozygotes over heterozygotes, potentially indicating the presence of null alleles. However, the estimated null allele frequency was low (frequency = 0.133) and I continued the paternity analysis with this locus included. The eight *E. leucoxylon* microsatellite loci provided an exclusionary power of 99.96% and no null alleles were detected amongst the 8 loci.

7.2.3   Statistical analyses

7.2.3.1   TWOGENER

I used the TWOGENER model (Smouse *et al.* 2001) to analyse patterns of pollen dispersal in the two populations. TWOGENER estimates the statistic Φ_{FT}, which measures the degree of differentiation of allelic frequencies among pollen pools sampled by several females in the population and thus tests the null hypothesis that pollen dispersal is panmictic (i.e. Φ_{FT}). However, the TWOGENER model does not perform well for species with a mixed mating system as a significant degree of selfing can upwardly bias the statistic Φ_{FT} (Burczyk & Koralewski 2005). Thus, I performed the TWOGENER analysis in two ways. Firstly, the analysis was performed on all progeny (which included selfed and outcrossed progeny) and, secondly, on outcrossed progeny only for each species. When selfed progeny were removed from the analysis several maternal trees had insufficient outcrossed progeny (n<5) to apply the TWOGENER model and these were removed from the analysis. Otherwise all sampled maternal trees were included in the analyses.

The number of effective males contributing to the estimate of Φ_{FT} can be estimated from the equation $N_{ep} = (2\Phi_{FT})^{-1}$ (Smouse *et al.* 2001). The number of "effective males" represents the number of individuals all contributing with equal probability and mating at random (panmictic population) that would yield the observed value of Φ_{FT}. The relationship between Φ_{FT} and pollen dispersal distance has also been derived for given dispersal curves (Austerlitz & Smouse 2001), allowing the development of several estimates of the average distance of pollen dispersal (Austerlitz & Smouse 2002). For the normal bivariate curve of pollen dispersal the average distance of pollen dispersal can be found from $\delta = \sigma\sqrt{\pi/2}$, where δ is the average distance of pollination and σ is the standard deviation of the distribution of pollen dispersal distances.

Φ_{FT} was calculated in GenAlEx v6.0 (Peakall & Smouse 2005) and the significance of the statistic determined by 99 permutations. Estimates of the average distance of pollen dispersal (δ) were also calculated in GenAlEx using estimates of average tree density and average distance to nearest conspecific (*distNC*) from Chapter 4. For *E. camaldulensis* paddock trees at Tungkillo, the average tree density was 1.03 trees.ha^{-1} and average *distNC* was 65m. For *E. leucoxylon* trees at Flaxley, the average tree density was 0.86 trees.ha^{-1} and average *distNC* was 72m. In addition, I estimated pollen dispersal distances for a range of tree density values, since in many situations effective tree density may vary from the measured adult density (e.g. when trees do not overlap in flowering period). Pollen dispersal distances were estimated in GenAlEx for effective densities (d_e) of 1.0 to 0.01 of the known adult density (d).

7.2.3.2   Paternity analysis

To directly estimate the average distance of pollen dispersal for each species paternity analysis was performed on offspring of *E. camaldulensis* and *E. leucoxylon* trees in the program *CERVUS* (Marshall *et al.* 1998). *CERVUS* performs paternity assignment based on maximum likelihood methods; the program calculates the likelihood that, given the offspring genotype, the candidate parent is the true parent versus the likelihood that the candidate parent is not the true parent. The statistical significance of the paternity assignment is assessed by randomly shuffling offspring genotypes to determine critical values of the log-likelihood statistic (Δ). For *E. camaldulensis* I performed the paternity simulation (used to assess statistical significance) using 10000

cycles, all potential pollen donors (n=25) as candidate parents and with a genotyping error rate of 0.001 (see below for discussion of error rates). Since I was not working with a closed system I assumed that not all candidate parents were sampled and that a proportion of successful matings could have been made with trees outside of the study area. Trees within the sampled paddock were relatively isolated from other trees (by ~100m), thus I assumed that the number of unsampled parents was low. For this reason I performed the paternity simulation assuming that only 90% of the potential parents had been sampled. This allows a proportion of seedlings to have unassigned paternity if they were unable to be assigned with confidence to the sampled parents.

It was more difficult to perform paternity analysis in the same manner on *E. leucoxylon* scattered trees. *Eucalyptus leucoxylon* has a patchy distribution at Flaxley and if paternity analysis was limited to all trees in one paddock the number of individuals sampled would have been ~5. In addition, *E. leucoxylon* is bird-pollinated and thus pollen dispersal is likely occurring over a very large area and not just restricted to a few trees. As such, I conducted paternity analysis on the offspring of all *E. leucoxylon* scattered trees sampled at Flaxley (trees occurred over an area of approximately 250ha) to see if I could detect any effective pollen dispersal events within the population. For the paternity simulation, I used the following parameters: Number of cycles = 10000, genotyping error rate = 0.001, proportion of parents sampled = 0.70, number of candidate parents = 32. I had sampled the majority of *E. leucoxylon* scattered trees on two properties at Flaxley but I did not sample roadside vegetation or trees on nearby properties, therefore it was prudent to choose a relatively low value for the proportion of candidate parents sampled. I chose 70% but it is also possible that this proportion is too high as the pollination neighbourhood of these trees may be much greater than predicted.

Genotyping errors (e.g. errors occurring due to mutation, null alleles, scoring errors, data handling errors) may bias paternity assignment (Morrissey & Wilson 2005). The program *CERVUS* can accommodate genotyping errors and allows a genotyping error rate to be set by the user. However, Morrissey & Wilson (2005) recommend setting the genotyping error rate to be lower than the "true" genotyping error rate to maximise the power of the paternity assignment. Though I did not specifically assess the genotyping error rate of my data set, as part of my normal genotyping quality control, any dubious

genotyping results were repeated and I confirmed that all seedling genotypes contained at least one maternal allele. Thus, I set the genotyping error rate at a low level (0.001) to maximise the number of correct paternity assignments. Following paternity assignment in *CERVUS*, I double-checked all assignments by comparing the genotypes of offspring, the known mother and the candidate parent. In some cases, the assignment of paternity was ambiguous due to missing data in the offspring or candidate parent – in these cases I rejected the paternity assignment and scored the offspring as "paternity unassigned".

7.3 Results

7.3.1 Pollen pool heterogeneity

TWOGENER analysis revealed that mating in both populations of paddock trees was not panmictic and that there was significant pollen pool structure (Table 7.2, Table 7.3). That is, for each species, pollen dispersal shows some spatial limitation and maternal trees are sampling from different local pollen pools. As demonstrated by Burczyk and Koralewski (2005), for species with a significant proportion of selfing the statistic Φ_{FT} can be artificially inflated and suggestive of very strong differentiation in the pollen pool sampled by different maternal trees. Mating system analysis (Chapter 6) showed that *E. camaldulensis* had a higher level of selfing than *E. leucoxylon* (~25% c.f. ~15%, respectively) and as Table 7.2 shows, for *E. camaldulensis* paddock trees Φ_{FT} was almost double when calculated for all progeny than for outcrossed seeds only. In contrast, *E. leucoxylon* trees showed only slight variation in Φ_{FT} between all progeny and outcrossed seeds only (Table 7.3). Nevertheless, it is useful to compare the TWOGENER results calculated from all progeny for both species as it reflects the realised mating system of each species. In this case, for both *E. camaldulensis* and *E. leucoxylon* paddock trees, Φ_{FT} indicates fairly high levels of pollen pool structure, suggesting that, on average, 2.5-3 males are contributing to the seed crop of maternal trees.

When only outcrossed seeds were included in the analysis, the patterns of pollen pool structuring were very different. Both species showed little variation in Φ_{FT} between cohorts. In *E. camaldulensis*, mating system analysis of the 2000 cohort (Chapter 6) suggested that the outcrossing rate was lower in this year but that the correlation of

paternity was also lower, leading to a greater diversity of males contributing to the seed crop. This was also reflected in the TWOGENER analysis with a lower Φ_{FT} and a higher estimate of the number of effective pollen donors (6.2 c.f. 4.1) for the year 2000 cohort of seedlings. When the two cohorts of seedlings were analysed together, there was a moderate level of pollen pool structuring, but an average of 5.2 effective males were contributing to the seed crop.

In contrast, in the mating system analysis of the previous chapter, *E. leucoxylon* trees showed very little variation in outcrossing rate or other mating system parameters between years, and this was also very much the case with the TWOGENER analysis of the outcrossed seeds. When both cohorts of seedlings (2001, 2003) were analysed together (Table 7.3), TWOGENER indicated much higher levels of pollen pool structuring than that detected for *E. camaldulensis* (Φ_{FT} = 0.15 vs 0.09, respectively). Thus, despite being bird-pollinated *E. leucoxylon* paddock trees showed much greater restriction of pollen dispersal than *E. camaldulensis* paddock trees. Indeed, only 3.2 effective males were estimated to contribute to the seed crop of maternal *E. leucoxylon* trees as opposed to 5.2 males for *E. camaldulensis* trees.

7.3.2   Mean pollen dispersal distance

The TWOGENER model estimates the average distance of pollen dispersal (δ) from genetic data based on a bivariate normal pollen dispersal curve, with input regarding the average density of adult trees and the average distance between conspecifics. Though the mating patterns of *E. camaldulensis* paddock trees varied slightly between cohorts, the estimated average distance of pollen dispersal was very similar between the two years (62-71m) (Table 7.4). When the two cohorts were analysed together the average distance of pollen dispersal for outcrossed seeds was 67m.

There were slight differences in the distribution of paddock trees at each study site and *E. leucoxylon* paddock trees tended to occur at lower densities than *E. camaldulensis* paddock trees. However, estimates of the average distance of pollen dispersal were remarkably similar between the two species. Again, there was little difference in pollination distance estimates between the two years and when the two cohorts were analysed together, the average distance of pollen dispersal was 62m (Table 7.4).

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Table 7.2: TWOGENER analysis of pollen pool heterogeneity for *E. camaldulensis* paddock trees.

Cohort	Source of variation	df	Estimated Variance	% of Variance	Φ_{FT}	P	N_{ep}
All progeny							
2000	Amongst mothers	29	0.473	20	0.200	0.010	2.5
	Within mothers	243	1.750	80			
2002	Amongst mothers	34	0.447	20	0.200	0.010	2.5
	Within mothers	307	1.833	80			
Years combined	Amongst mothers	47	0.410	18	0.180	0.010	2.8
	Within mothers	567	1.872	82			
Outcrossed progeny only							
2000	Amongst mothers	29	0.178	8	0.081	0.010	6.2
	Within mothers	151	2.033	92			
2002	Amongst mothers	34	0.266	12	0.121	0.010	4.1
	Within mothers	225	1.927	88			
Years combined	Amongst mothers	47	0.212	10	0.096	0.010	5.2
	Within mothers	393	2.006	90			

Table 7.3: TWOGENER analysis of pollen pool heterogeneity for *E. leucoxylon* paddock trees

Cohort	Source of variation	df	Estimated Variance	%	Φ_{FT}	P	N_{ep}
All progeny							
2001	Amongst mothers	22	0.53	19	0.185	0.01	2.7
	Within mothers	185	2.33	81			
2003	Amongst mothers	22	0.447	18	0.176	0.01	2.8
	Within mothers	192	2.227	82			
Years combined	Amongst mothers	27	0.47	16	0.165	0.01	3.0
	Within mothers	395	2.37	84			
Outcrossed progeny only							
2001	Amongst mothers	20	0.451	16	0.165	0.01	3.0
	Within mothers	143	2.281	84			
2003	Amongst mothers	20	0.451	16	0.162	0.01	3.1
	Within mothers	156	2.334	84			
Years combined	Amongst mothers	26	0.425	15	0.154	0.01	3.2
	Within mothers	318	2.330	85			

Thus, while *E. camaldulensis* has higher levels of selfing, the patterns of outcrossed pollination are very similar to that of *E. leucoxylon*, despite the difference in mobility between their main pollinators. In both species, the average distance of pollen dispersal is very close to the average distance between conspecifics which tends to suggest that paddock trees are most likely to be frequently mating with their nearest neighbours.

The TWOGENER model estimates the average distance of pollen dispersal from known adult density, however, in many situations adult tree density may not reflect "effective" tree density. This may arise when there are differences in the phenological overlap of trees such that only a few individuals are reproductively compatible at any one time. TWOGENER also provides for modelling the pattern of pollen dispersal when effective tree density (d_e) differs from adult density (d). As Figure 7.1 shows, *E. camaldulensis* and *E. leucoxylon* paddock trees respond similarly to changes in the proportion of reproductive adults. When effective tree density is one tenth that of the known adult tree density, the average distance of pollen dispersal increases to approximately 135-140m for both species. Flowering in *E. camaldulensis* trees at Tungkillo is relatively synchronous, occurring over a 3-4 week period, though trees may reach peak flowering at different times during this period. On the other hand, *E. leucoxylon* has a longer flowering period and may spot-flower throughout the year. Thus, *E. leucoxylon* trees are potentially likely to experience a greater difference in effective density relative to known tree density than *E. camaldulensis* trees.

Table 7.4: Average distance of pollen dispersal (δ) for outcrossed progeny of *E. camaldulensis* and *E. leucoxylon* paddock trees.

$N_{mothers}$ = Number of maternal trees sampled
distNC = Distance to nearest conspecific

Cohort	Φ_{FT}	$N_{mothers}$	σ	δ (m)
E. camaldulensis				
Adult density = 1.03 trees.ha^{-1}				
Mean *distNC* = 65m				
2000	0.081	30	0.565	71
2002	0.121	35	0.495	62
Years combined	0.096	48	0.535	67
E. leucoxylon				
Adult density = 0.86 trees.ha^{-1}				
Mean *distNC* = 72m				
2001	0.165	21	0.481	61
2003	0.162	21	0.484	61
Years combined	0.154	27	0.497	62
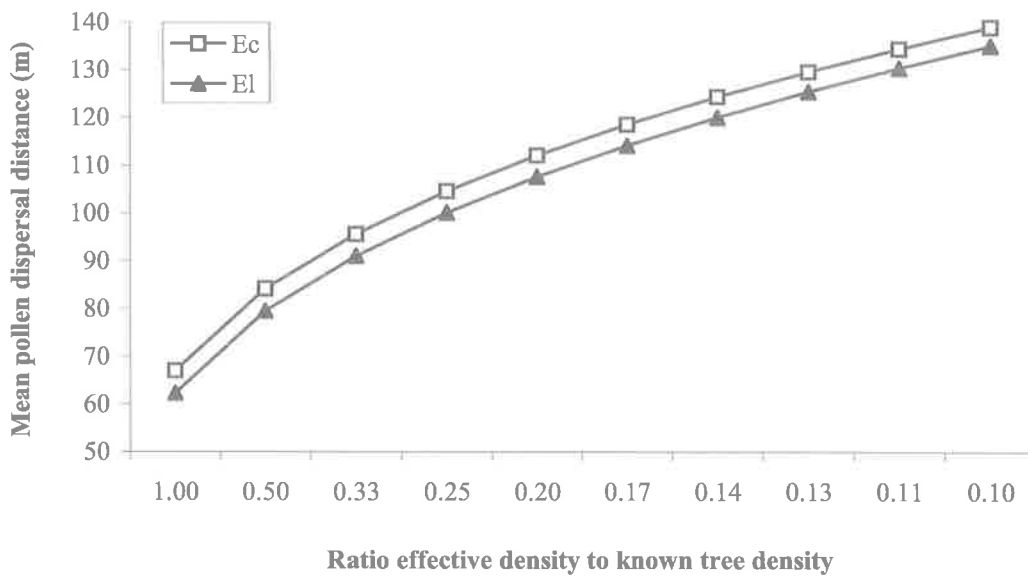
Figure 7.1: Change in average distance of pollen dispersal (δ) as effective tree density declines.

Ec = *Eucalyptus camaldulensis;* El = *Eucalyptus leucoxylon*

7.3.3   Paternity analysis – estimating the "real" pollen dispersal curve

7.3.3.1   Paternity analysis of E. camaldulensis *paddock trees*

I performed paternity analysis on offspring from seven *E. camaldulensis* trees occurring in a single paddock at Tungkillo. 97 seedlings were sampled, and of these 62 were derived from outcrossing events, representing an outcrossing rate of 63%. The program *CERVUS* assigned paternity to 86% of seedlings with 80% confidence, but only 49% of seedlings could be assigned paternity with 95% confidence (Table 7.5). 14% of seedlings were unable to be assigned paternity with confidence. These seedlings potentially represent the number of matings that have occurred with trees outside of the study area (i.e. unsampled males). However, as I could not assume my data set was error free a small proportion of unassigned paternity may be due to parent-offspring mismatches caused by genotyping error or missing data.

Table 7.5: Summary of *CERVUS* paternity assignment results for *E. camaldulensis* paddock trees.

Level	Confidence (%)	Delta Criterion	Tests	Success Rate
Strict	95	1.09	48	49%
Relaxed	80	0.00	83	86%
Unresolved			14	14%

The results of the paternity analysis of *E. camaldulensis* trees are summarised in Table 7.6. For most of the sampled trees I was able to assign paternity to the majority of outcrossed seedlings, however for two trees (Ec3347, Ec3342) a significant proportion of seedlings were unable to be assigned with confidence to candidate parents within the paddock. Of the outcrossed seedlings for which paternity could be assigned, the average distance of pollen dispersal within the paddock was 299m. The mean pollination distance for individual trees ranged from 225m – 501m, with some trees receiving pollen from trees up to 650m distant. The mean nearest neighbour distance of trees within the paddock was 98m so clearly pollen dispersal occurred over a very large area and trees are not limited to mating with their nearest neighbours. Again, these pollen dispersal distances may be regarded as lower estimates of the maximum dispersal distances, as trees from outside the study area potentially fathered a number of seedlings. When selfing events were accounted for (pollen dispersal distance = 0), the average distance of pollen dispersal within the paddock was 181m.

On average, five male parents contributed to the seedling crop of the sampled paddock trees, but for individual trees the number of male parents ranged from 1-10. A number of correlated matings were detected (e.g. for maternal trees Ec3346 and Ec3349), however, when multiple matings occurred, the same father sired only 2-4 offspring. This tends to support the results from the previous mating system analysis, which suggested a low rate of correlated paternity for low density paddock trees.

Figure 7.2 clearly illustrates the spatial scale of mating patterns of individual trees. As indicated above, only a few trees mated with their nearest neighbours (e.g. Ec3349, Ec3346), whereas the majority of matings occurred with trees over a very large spatial scale. In addition, correlated matings were not necessarily with nearest neighbours either; for example Ec3342 had 3 seedlings sired by an individual located almost 600m distant.

It is hard to determine the shape of the pollen dispersal curve (Figure 7.3) constructed from data on successful mating events within the paddock of *E. camaldulensis* trees. Pollen dispersal has frequently shown a leptokurtic distribution for many species (e.g. Krauss 2000; Hardy *et al.* 2004; Robledo-Arnuncio & Gil 2005), exhibiting a high frequency of matings at close distance with a slow decline in mating frequency to large distances. In this study, *E. camaldulensis* trees were infrequently mating with their nearest neighbours, thus the peak in pollen dispersal distance occurred around 200-250m, and with a slow decline in the frequency of mating with increasing distance. Increasing the number of seedlings sampled per individual would likely have produced a more robust estimate of the distribution of pollen dispersal distances.

Twenty out of the 25 candidate parents in the paddock contributed pollen to the seedling pool of the 7 mother trees sampled (Figure 7.4). The male contribution was relatively evenly spread across the population, with only three males contributing to 5 or more seedlings and 8 males contributing only one seedling. Of the seven maternal trees, two individuals (Ec3347 and Ec3345) did not contribute as males to the seedling pool.

Table 7.6: Results of paternity analysis of *E. camaldulensis* paddock trees.

[a] Superscripts denote confidence levels of paternity assignment: + = 80% confidence level, * = 90% confidence level.
[b] Average pollen dispersal distance of outcrossed seeds.
[c] Average pollen dispersal distance for all seeds (i.e. including selfing)

Maternal parent	No. selfed seeds	No. outcrossed seeds	No. paternity assigned	No. paternity unassigned	Paternal parent[a]	Inter-mate Distance (m)	No. mating partners	Mean pollination distance[b] (m)	Mean pollination distance[c] (m)
Ec3347	13	8	3	5	Ec6478 $^+$	280	3	225	42
					Ec6485 *	237			
					Ec6489 $^+$	159			
Ec3346	2	16	14	2	Ec3342 *	551	10	279	244
					Ec3345 $^+$	363			
					Ec3349 $^+$	173			
					Ec3349 *	173			
					Ec3349 $^+$	173			
					Ec3349 $^+$	173			
					Ec5625 $^+$	396			
					Ec6479 $^+$	344			
					Ec6485 $^+$	275			
					Ec6486 $^+$	286			
					Ec6487 *	188			
					Ec6491 $^+$	209			
					Ec6494 *	303			
					Ec6494 $^+$	303			
Ec3349	1	19	17	2	Ec3348 *	154	10	257	242
					Ec3348 $^+$	154			
					Ec3350 *	137			
					Ec6478 $^+$	486			
					Ec6481 $^+$	533			
					Ec6481 *	533			

					Ec6485[+]	139			
					Ec6486[+]	175			
					Ec6488[+]	208			
					Ec6488[+]	208			
					Ec6488[+]	209			
					Ec6490[+]	320			
					Ec6490[+]	320			
					Ec6494[+]	314			
					Ec6495[*]	158			
					Ec6495[+]	158			
					Ec6495[+]	158			
Ec3345	12	2	2	0	Ec3348[*]	341	1	341	48
					Ec3348[+]	341			
Ec3342	1	8	5	3	Ec6482[*]	652	3	501	418
					Ec6484[+]	123			
					Ec6488[+]	577			
					Ec6488[*]	577			
					Ec6488[+]	577			
Ec3350	5	3	2	1	Ec6490[+]	305	2	249	71
					Ec6495[+]	192			
Ec3348	1	6	6	0	Ec3342[*]	425	6	239	205
					Ec3349[*]	154			
					Ec6478[+]	415			
					Ec6491[+]	206			
					Ec6494[+]	167			
					Ec6495[*]	66			
Total	**35**	**62**	**49**	**13**	**Average**	**287m**	**5**	**299m**	**181m**

a. Ec3347
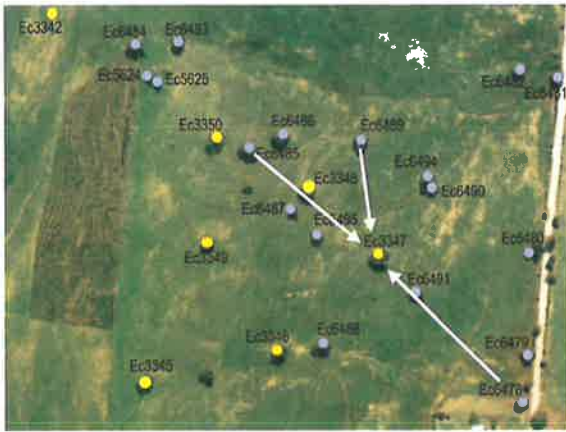
b. Ec3346
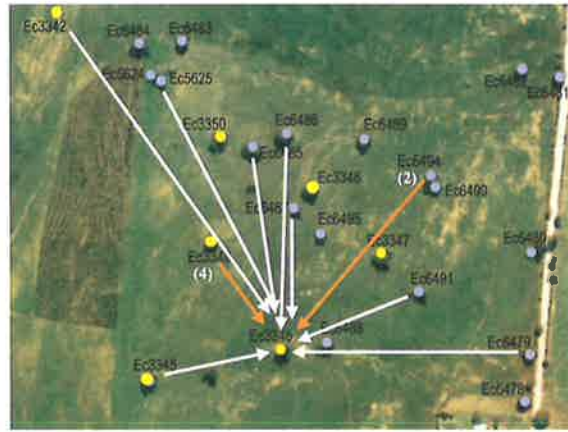
c. Ec3349
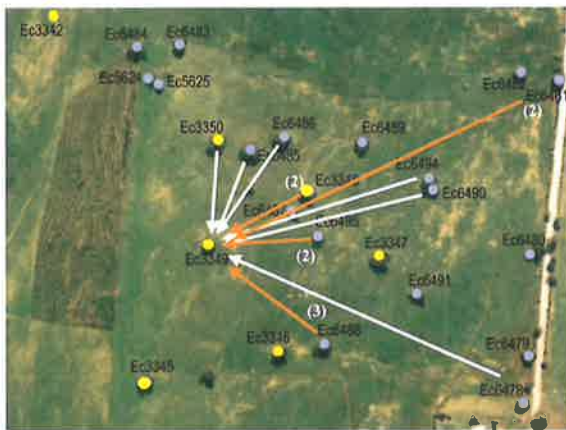
d. Ec3342
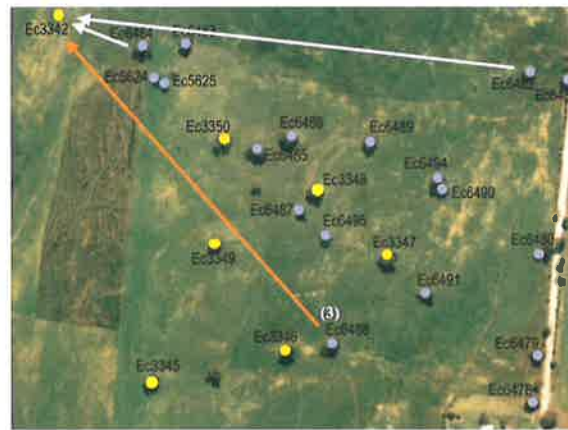
e. Ec3350
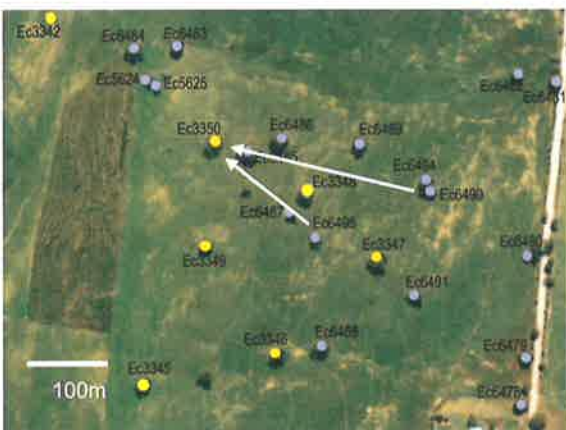
f. Ec3348
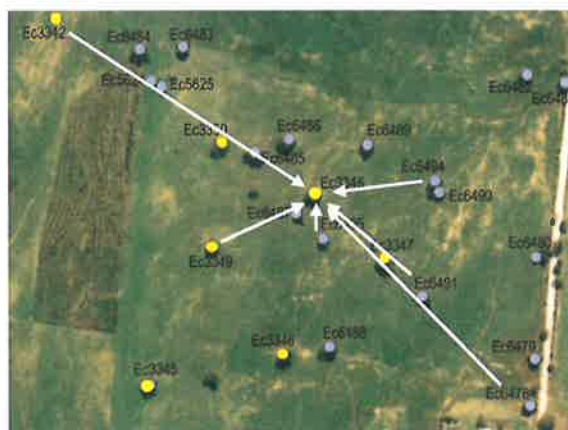
Figure 7.2: Mating patterns of individual *E. camaldulensis* paddock trees determined by paternity analysis

White arrows represent single matings; orange arrows represent multiple matings, with the number of seeds sired by the male parent in brackets. Note the scale bar to the bottom left of figure e.
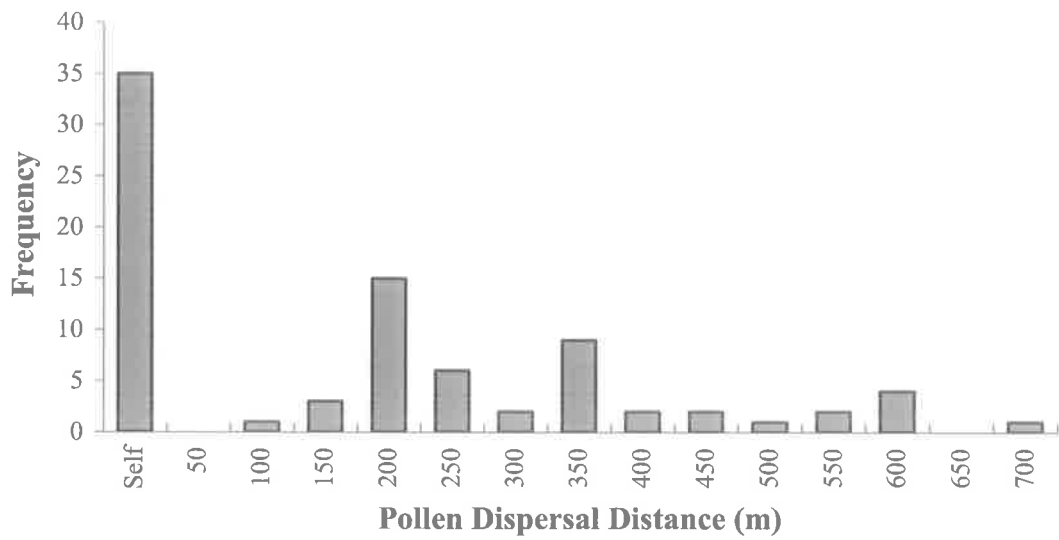
Figure 7.3: Histogram of pollen dispersal distances for successful matings of a subset of *E. camaldulensis* paddock trees determined by paternity analysis.
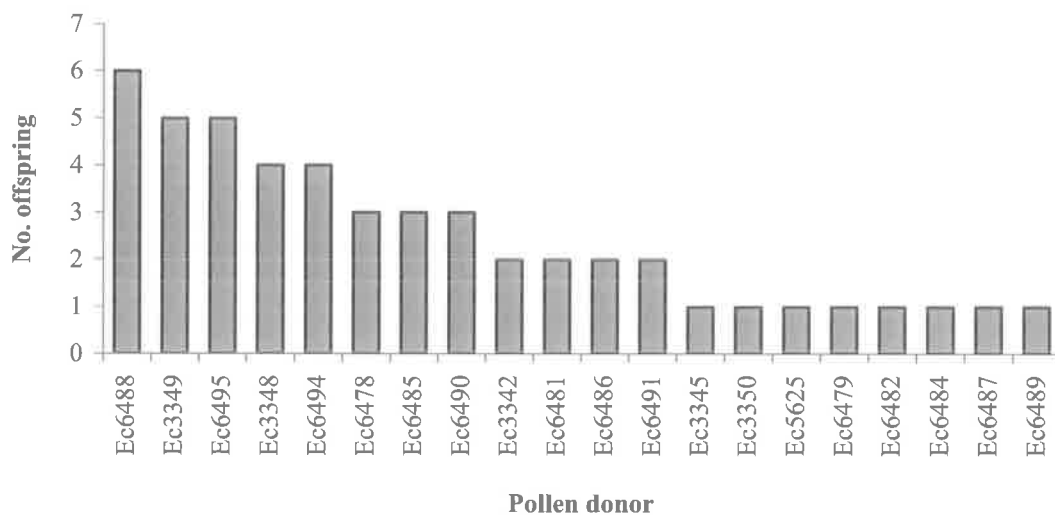
Figure 7.4: Number of offspring sired by individual male *E. camaldulensis* parents determined by paternity analysis

7.3.3.2   Paternity analysis of E. leucoxylon *paddock trees*

Paternity analysis of *E. leucoxylon* was performed on progeny from all paddock trees sampled at Flaxley, and including all paddock trees as candidate parents. *CERVUS* assigned paternity to 69% of seedlings with at least 80% confidence (Table 7.7). However, after carefully checking the paternity assignments I noted a number of obvious parent-offspring mismatches that had occurred, despite a stringent genotyping error rate used during the *CERVUS* analysis. For a number of these seedlings I rejected paternity assignments due to missing data, and overall I assigned paternity with at least 80% confidence to 46% of seedlings. As mentioned previously, it is likely that a high proportion of seedlings where paternity was unable to be assigned were due to matings with unsampled individuals.

Table 7.7: Summary of *CERVUS* paternity assignment results for *E. leucoxylon* paddock trees.

Level	Confidence (%)	Delta criterion	Tests	Success Rate
Strict	95	1.64	197	47%
Relaxed	80	0.00	292	69%
Unresolved			130	31%

Of 359 seedlings analysed, 95 seedlings were due to selfing events (outcrossing rate = 74%). Of the remaining 259 outcrossed seedlings, paternity could only be assigned with confidence to 82 offspring (Table 7.8). Again, the paternity analysis tended to support the results of the mating system analysis performed in the previous chapter. I found that low density paddock trees tended to have a higher correlation of paternity (higher than that for *E. camaldulensis* paddock trees), and indeed paternity assignment revealed numerous examples of multiple matings (e.g. for maternal trees El5606, El5607, El5619). The majority of effective pollen dispersal events occurred over 100-500m but I detected a number of mating events up to 1.5km and even a single mating event between trees located 2.3km apart (Table 7.8). For the outcrossed seedlings for which paternity could be assigned, the average distance of outcrossed pollen dispersal within the population was 522m, an average of 590m per tree. However, as the distribution of pollen dispersal distances shows (Figure 7.6), pollen dispersal appeared to be almost bimodal with the majority of matings occurring between trees 100-500m apart, but also a substantial number of matings occurring separated by 1-2.5km. This is

potentially consistent with bird pollinator behaviour as birds may frequently forage on a local scale but also make long distance flights between patches. Potentially the median distance (rather than the mean) of outcrossed pollen dispersal better describes the pattern of pollen dispersal in *E. leucoxylon* paddock trees. The median distance of outcrossed pollen dispersal was 184m. Across the entire study site, the average distance to nearest conspecific was 72m, so quite clearly pollen dispersal is occurring over a much greater area. When selfing events were included, the average distance of pollen dispersal was 239m per tree.

Importantly, when mating events are visualised on a landscape scale (Figure 7.5) there appears to be high levels of connectivity across the population of *E. leucoxylon* paddock trees. In addition, a number of gene flow events were detected between isolated paddock trees and trees located in high density patches (for example, for trees El5618, and El5621). This supports the notion that paddock trees may facilitate gene flow between more isolated patches of vegetation.

Though there were many trees for which a large number of offspring could not be assigned paternity, the average number of mating partners per tree was 2.3 individuals. The male contribution of trees to sampled offspring appeared to be relatively even (Figure 7.7). Two trees (El5608 and El6492) contributed to a large number of offspring (16 and 11 seedlings, respectively) and several trees contributed to 5-6 seedlings. However, at least a proportion of these were due to multiple matings with near neighbours (especially for El5608). As was reported for *E. camaldulensis,* a high proportion of *E. leucoxylon* trees contributed to only one or two seedlings.

Table 7.8: Results of paternity analysis of *E. leucoxylon* paddock trees.

[a] Superscripts denote confidence levels of paternity assignment: + = 80% confidence level, * = 90% confidence level.
[b] Average pollen dispersal distance of outcrossed seeds.
[c] Average pollen dispersal distance for all seeds (i.e. including selfing)

Maternal parent	No. selfed seeds	No. outcrossed seeds	No. paternity assigned	No. paternity unassigned	Paternal parent[a]	Inter-mate Distance (m)	No. mating partners	Mean pollination distance[b] (m)	Mean pollination distance[c] (m)
El 5601	10	5	3	2	El 5623[+]	925	3	1088	251
					El 5602[*]	38			
					El 6456[*]	2300			
El 5602	0	3	1	2	El 5601[*]	38	1	38	38
El 5606	1	19	4	15	El 5612[+]	1448	2	576	461
					El 5608[*]	285			
					El 5608[*]	285			
					El 5608[*]	285			
El 5607	0	21	15	6	El 5606[*]	153	4	306	306
					El 5606[*]	153			
					El 5608[+]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5608[*]	147			
					El 5618[+]	1581			
					El 6492[*]	1086			
El 5608	10	10	5	5	El 5613[*]	1053	4	1125	375
					El 5618[+]	1467			

					El 6455*	1155			
					El 6492+	975			
					El 6492+	975			
El 5609	2	13	2	11	El 5623*	250	2	916	458
					El 6454*	1582			
El 5610	1	9	2	7	El 5609*	22	2	63	42
					El 5612+	104			
El 5611	2	7	1	6	El 5610*	40	1	40	40
El 5612	1	9	3	6	El 5609*	86	3	126	95
					El 5620*	110			
					El 5623*	182			
El 5613	0	10	2	8	El 5616+	97	2	171	171
					El 5623+	245			
El 5618	7	20	1	19	El 5616*	503	1	503	63
El 5619	0	20	9	11	El 5602+	1180	6	361	361
					El 5607+	1534			
					El 5609+	71			
					El 5609+	71			
					El 5618*	87			
					El 5618*	87			
					El 5618*	87			
					El 5620*	40			
					El 5621*	89			
El 5620	8	12	2	10	El 5609*	34	2	149	30
					El 5623*	263			
El 5621	17	3	1	2	El 6493*	1641	1	1641	92
El 5622	0	20	5	15	El 5603*	455	2	691	691
					El 5603+	455			
					El 5603+	455			
					El 5603*	455			
					El 5608*	1636			
El 5623	9	7	4	3	El 5616*	332	4	612	188
					El 5618+	269			

					El 5621*	185			
					El 6455+	1660			
El 6451	0	17	3	14	El 6492*	96	2	115	115
					El 6492+	96			
					El 6493*	148			
El 6454	4	11	6	5	El 6492*	176	1	176	106
					El 6492+	176			
					El 6492*	176			
					El 6492*	176			
					El 6492*	176			
					El 6492*	176			
El 6455	8	15	6	9	El 6493*	310	3	695	298
					El 6493*	310			
					El 6493*	310			
					El 6493*	310			
					El 5615+	1495			
					El 5613*	1432			
El 6457	1	9	2	7	El 6455+	64	2	617	411
					El 5608+	1169			
El 6458	2	8	2	6	El 5615*	1448	2	758	379
					El 6455+	68			
El 6492	5	5	1	4	El 5616*	1280	1	1280	213
El 6493	7	6	2	4	El 5615*	1479	2	1529	340
					El 5609+	1578			
Total	**95**	**259**	**82**	**177**	**Average**	**522m**	**2.3**	**590m**	**239m**
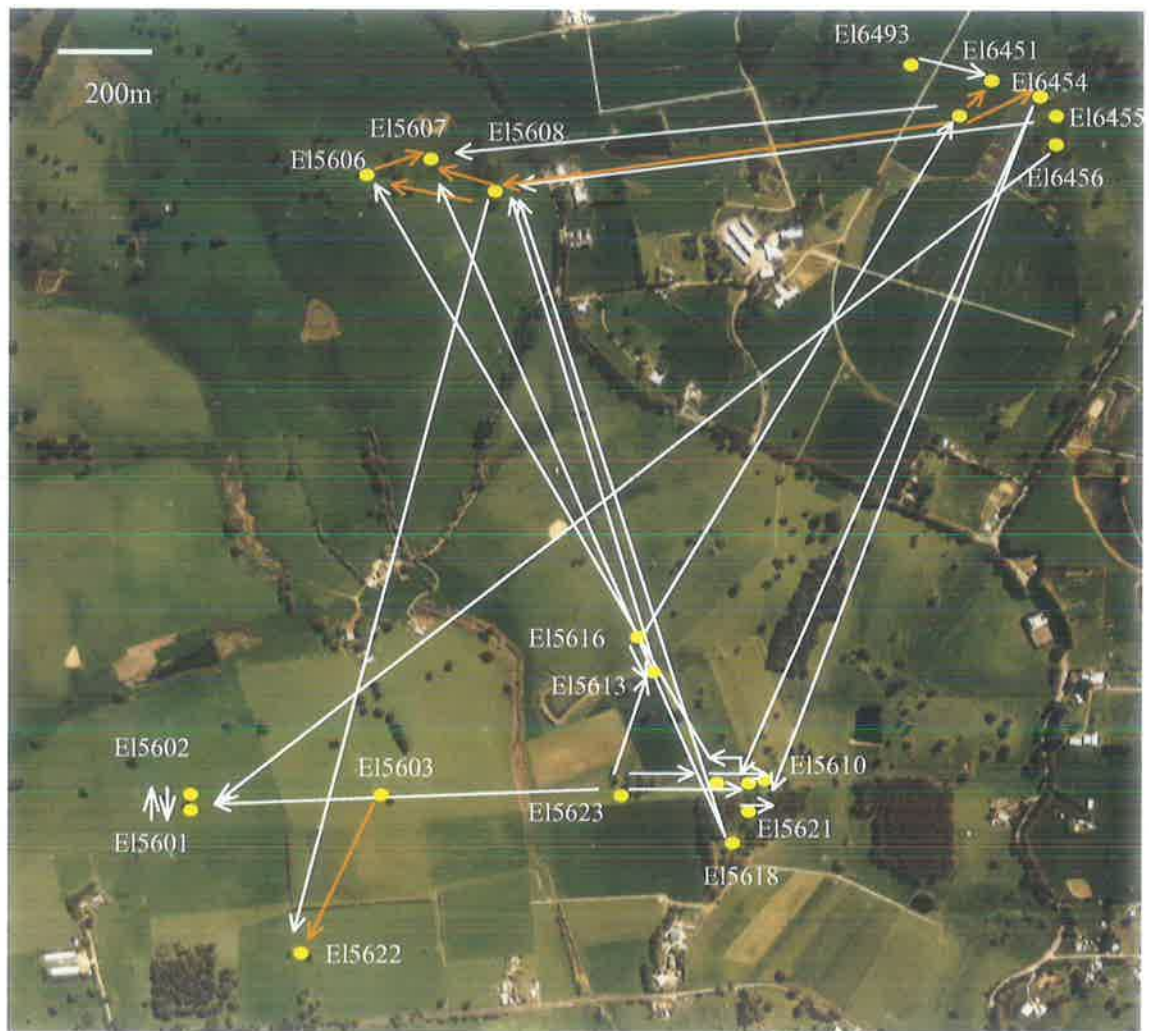
Figure 7.5: Examples of the mating patterns of select *E. leucoxylon* paddock trees (n=14 maternal trees) determined by paternity analysis.

White arrows represent single mating events; orange arrows represent multiple mating events (see Table 7.8 for the number of mating events per individual). Note the scale bar in the upper left of the image.
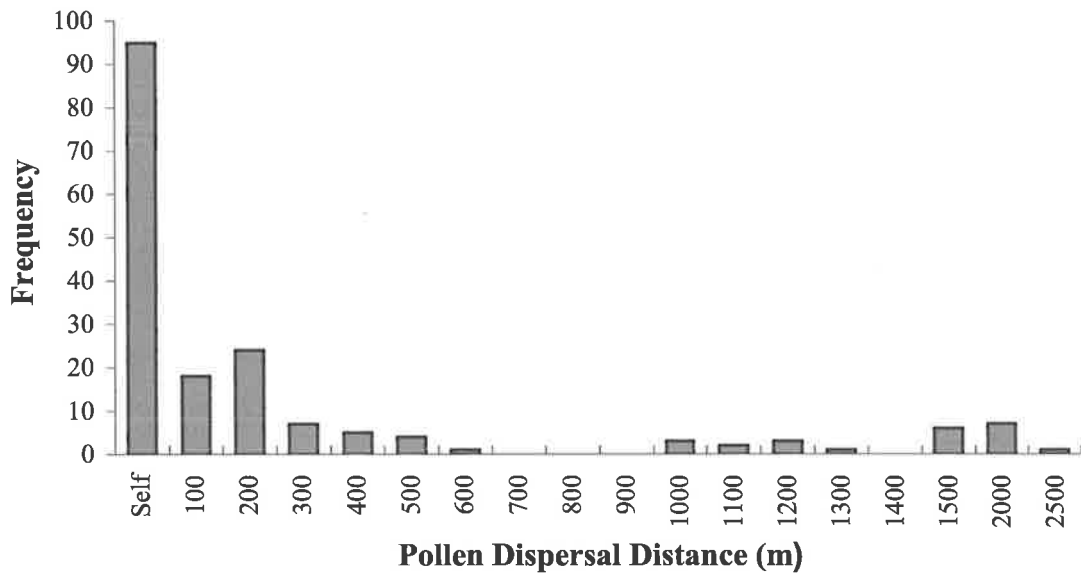
Figure 7.6: Histogram of pollen dispersal distances for successful matings of *E. leucoxylon* paddock trees determined by paternity analysis.
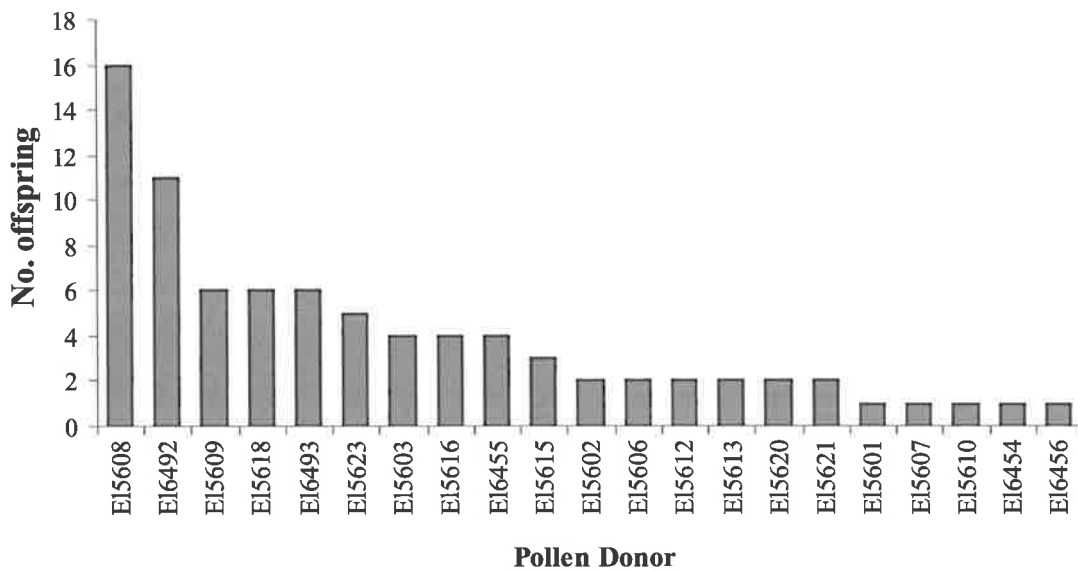
Figure 7.7: Number of offspring sired by individual male *E. leucoxylon* parents determined by paternity analysis

7.4 Discussion

As opposed to traditional approaches to measuring pollen dispersal in plant populations (e.g. tracking chemically-labelled pollen, pollinator observations), molecular genetic approaches measure *effective* pollen dispersal and can provide revealing insights into contemporary patterns of pollen gene flow. In this study I employed two differing approaches to estimate patterns of pollen dispersal in *E. camaldulensis* and *E. leucoxylon* paddock trees, each species having suffered enormous declines in plant density. As suggested by mating system analyses on these trees, the agricultural environment is far from a 'biological desert': paddock trees are reproductively viable and, on the whole, pollinator activity is sufficient to maintain population outcrossing rates of paddock trees. Indeed, both the TWOGENER analysis and paternity analysis revealed surprisingly high levels of pollen gene flow in both species of paddock trees.

The TWOGENER approach was introduced by Smouse *et al.* (2001) and has proved useful for estimating patterns of pollen dispersal in populations where paternity analysis methods may not be feasible (for example, in continuous populations). It essentially describes the degree of overlap in pollen pools sampled by female trees and, thus, tests the degree of deviation from a model of random, global pollen dispersal (i.e. panmixia). In both *E. camaldulensis* and *E. leucoxylon* paddock tree populations I detected significant pollen pool structure. When the TWOGENER model was applied to genetic data from all progeny of paddock trees (i.e. including both selfed and outcrossed progeny), I found quite strong pollen pool genetic structure, especially for *E. camaldulensis* trees (Φ_{FT} = 0.20 and 0.17, for *E. camaldulensis* and *E. leucoxylon*, respectively). This is to be expected, as significant levels of selfing will act to upwardly bias Φ_{FT} since the predominance of maternal alleles in the estimates of paternal allele frequencies is likely to lead to greater differentiation in pollen pools between females.

Indeed, quite a different picture emerged when only outcrossed offspring were included in the TWOGENER analysis. While Φ_{FT} did not change significantly for *E. leucoxylon*, due to the low level of selfing in this species, it did indicate a lower estimate of the degree of pollen structure (Φ_{FT} = 0.15 c.f. 0.17) and consequently a greater degree of pollen dispersal. Most surprisingly, analysis of only the outcrossed progeny of *E. camaldulensis* paddock trees revealed very low levels of pollen pool structure, half that

found in the analysis of all offspring (Φ_{FT} = 0.10 c.f. 0.20). Thus, while *E. camaldulensis* trees experience significant amounts of selfing, of the outcrossed component of mating, *E. camaldulensis* trees experience high levels of pollen gene flow.

Intriguingly, these estimates of Φ_{FT} suggest that *E. camaldulensis* trees, which are insect-pollinated, may be experiencing higher levels of pollen gene flow than *E. leucoxylon* trees, which are primarily bird-pollinated. This is in contrast to expectations based upon the relative mobility of insect versus bird pollinators. In fact, estimates of the number of effective pollen donors (N_{ep}) indicated that *E. camaldulensis* paddock trees mated with a greater number of individuals than *E. leucoxylon* paddock trees (N_{ep} = 5.2 c.f. 3.2, respectively). This was also suggested by the mating system analysis and estimates of N_{ep} are very similar to those calculated in MLTR ($1/r_p$ = 5.7 c.f. 3.8, respectively). However, it must be noted that these estimates of the number of mating partners are based on the concept of the "idealised" male, i.e. males are mating randomly and all males have equal contribution. In reality, N_{ep} is frequently an underestimate of the "real" number of mating partners due to the effect of unequal contribution of males (P.E. Smouse, pers. comm.).

TWOGENER analysis can also be used to translate Φ_{FT} into an estimate of the average distance of pollen dispersal, based upon the average density of trees in the landscape and the average distance between individuals (Austerlitz & Smouse 2002). Based upon a bivariate normal distribution of pollen dispersal distances, I estimated the average pollination distance of *E. camaldulensis* trees at Tungkillo to be 67m. *Eucalyptus leucoxylon* trees occurred at lower densities and with a greater average nearest neighbour distance, but the estimate of the average distance of pollen dispersal was 62m in this species, indicating slightly more restricted pollen dispersal than for *E. camaldulensis*. The average nearest neighbour distance for *E. camaldulensis* trees was 65m and 72m for *E. leucoxylon* so these results indicate that paddock trees are potentially mating most frequently with their nearest neighbours. Overall, both of these estimates fall within the range of average pollen dispersal distances reported for other *Eucalyptus* species (e.g. 35m, Chaix *et al.* 2003; up to 100m, Barbour *et al.* 2005).

"Real-time" patterns of pollen-mediated gene flow can be directly estimated using paternity assignment methods. The paternity assignment approach requires that all candidate parents (or at least the majority) are known, and for many plant populations this may be difficult to achieve. In this study, I performed paternity analysis on offspring from a subset of *E. camaldulensis* trees, using all trees that occurred in a single paddock at Tungkillo as candidate parents. I made the assumption that the majority of pollen gene flow would occur amongst the trees within the paddock, but paternity analysis revealed that approximately 25% of matings occurred with trees outside of the prescribed area. Of the outcrossed offspring that were assigned paternity to trees within the paddock, the average distance of pollen gene flow was 299m and the maximum distance recorded was 648m. Similarly, paternity analysis, performed on the entire population of *E. leucoxylon* paddock trees at Flaxley, detected many long distance mating events (up to 2.3km) and the average distance of pollen dispersal was 590m. However, this estimate is certainly biased by the number of long distance mating events and the median distance of pollen dispersal (184m) may better describe the patterns of pollen dispersal for *E. leucoxylon* paddock trees. Quite clearly though, these estimates of average pollen dispersal distances in *E. camaldulensis* and *E. leucoxylon* paddock trees are several orders of magnitude greater than the average pollen dispersal distance estimated by the TWOGENER analysis (62-67m) and that suggested by other studies of pollen dispersal in *Eucalyptus* species.

The TWOGENER analysis also suggested that *E. camaldulensis* and *E. leucoxylon* paddock trees may be most frequently mating with their nearest neighbours since the average distance of pollen dispersal was very close to the average nearest neighbour distances at each study site. Many of the sampled *E. leucoxylon* paddock trees had matings with their nearest neighbours, but long distance mating events were also frequent. However, for *E. camaldulensis* the paternity analysis showed that only a few mating events were between nearest neighbours and were more often between trees located 2-3 trees distant or even very long distance gene flow events. These patterns of mating for *E. camaldulensis* trees may potentially reflect a non-overlap in flowering times between closely located individuals, although I have observed that, in general, in the population of *E. camaldulensis* trees at Tungkillo, closely located trees overlap in flowering period. Alternatively, these patterns may reflect differences in pollinator behaviour when floral resources change throughout the flowering period. It is likely

that a single *E. camaldulensis* paddock tree in peak flowering would provide enough resources for any one insect pollinator on a foraging bout and, thus, there would be no impetus to move between trees. Instead, insect pollinators may only move between trees when floral resources are limited, for example at the start or end of the flowering period. Bird pollinators may also act in a similar manner, making long distance movements when floral resources are limited but due to their larger body size, during peak flowering times bird pollinators may still need to move between trees in order to satisfy their energetic requirements. Interestingly, a number of other studies involving insect-pollinated paddock trees also reported that trees were not frequently mating with their nearest neighbours (Chase *et al.* 1996; Dick 2001; White *et al.* 2002).

The average number of mating partners determined by paternity analysis reflected the patterns suggested by both the TWOGENER analysis and mating system analyses. Across the seven maternal *E. camaldulensis* trees sampled in this study, trees had an average of 5 mating partners, and only a few correlated matings were detected. In contrast, I identified an average of 2.3 mating partners for *E. leucoxylon* paddock trees and a greater number of correlated matings were detected for *E. leucoxylon* trees. In *E. camaldulensis* there was a relatively even contribution of males, most trees contributed to 2 or more seedlings and many trees contributed to single mating events. In contrast, a number of *E. leucoxylon* males appeared to have high reproductive success contributing to more than 10 offspring each. However, the majority of these were due to correlated mating events between single trees rather than a greater spread across many maternal trees.

Quite surprisingly, analysis of the patterns of pollen dispersal for *E. camaldulensis* paddock trees revealed pollen gene flow over a much greater spatial scale than anticipated for this predominantly insect-pollinated species. As mentioned previously, the introduced honeybee *Apis mellifera* is a frequent floral visitor of *E. camaldulensis* paddock trees, collecting both pollen and nectar. The larger body size and greater flight capabilities of honeybees, in comparison to native bees, may potentially account for the large observed pollen dispersal distances in *E. camaldulensis* since honeybees have been found to be capable of making foraging flights of up to 9km (Beekman & Ratnieks 2000). However, honeybees may actually play an alternative role in the observed patterns of pollen dispersal. In contrast to native bee species, which are solitary or

primitively eusocial, European honeybees are social insects and may form colonies of hundreds of individuals. Individual honeybees generally forage on a particular food source then return to the nest to deposit pollen or nectar when sufficient amounts have been collected. Since any particular eucalypt paddock tree contains more pollen or nectar than required for any one honeybee, it seems unlikely that honeybees would frequently move between trees on foraging bouts, thereby effecting outcrossed pollen transfer. Instead, there is potential for individuals within a nest to pick up pollen that has been transferred there by many individual bees from many different trees and then to deposit this mixed pollen load onto a single tree with the next foraging bout. This mechanism could explain the low level of correlated matings and the high diversity of mating partners detected for *E. camaldulensis* paddock trees.

Nonetheless, on a landscape-scale the observed patterns of pollen dispersal suggest that there is indeed high inter-connectivity between individuals in both *E. camaldulensis* and *E. leucoxylon* paddock tree populations. In addition, the landscape-scale paternity analysis of *E. leucoxylon* paddock trees showed that there were a number of mating events between isolated paddock trees and trees located in high-density patches. While I did not perform the same analysis for *E. camaldulensis* trees, the spatial scale over which pollen dispersal occurs in this species suggests that there would also be connectivity between isolated trees and trees in remnant patches. Thus, the presence of paddock trees in the landscape may indeed facilitate gene flow between small, isolated remnant patches and therefore ameliorate the effects of increased inbreeding and genetic drift to which small populations are susceptible (Young *et al.* 1996).

7.4.1 Comparison of genetic methods for estimating patterns of pollen dispersal

This study demonstrated the utility and also the drawbacks of using each of the two genetic approaches to estimate patterns of pollen dispersal in plant populations. Firstly, results from both the TWOGENER and paternity analyses were surprisingly concordant with the general mating patterns, for both *E. camaldulensis* and *E. leucoxylon* trees, that were suggested by the mating system analyses performed in the previous chapter. Both the TWOGENER analysis and the paternity analyses confirmed that mating was non-random in both populations of paddock trees and that maternal trees were sampling from different, potentially localised, pollen pools.

However, the TWOGENER analysis appeared to substantially underestimate the average distance of pollen dispersal in both paddock tree populations. TWOGENER estimates of the average distance of pollen dispersal were several orders of magnitude lower than that determined by paternity analysis. There may be several reasons for this apparent mismatch, which may be due to both methodological problems or to the biological properties of pollen dispersal. Firstly, TWOGENER uses a modelling approach based on an idealised distribution of pollen dispersal distances. In this study, pollen dispersal distances were estimated from a bivariate normal curve of pollen dispersal. Normal curves have been found to provide the most conservative estimates of pollen dispersal distances, since the tail of the distribution is fat-tailed and this may not effectively represent the long tail of strongly leptokurtic pollen dispersal (Austerlitz *et al.* 2004). For example, Burczyk & Koralewski (2005) have shown that TWOGENER estimates of the average distance of pollen dispersal are underestimated by approximately 50% when plants experience a significant amount of long-distance dispersal. Paternity analysis of *E. camaldulensis* and *E. leucoxylon* revealed the potential for long distance dispersal in each species and it may have been more appropriate to explore a range of pollen dispersal curves (exponential, exponential power, Weibull), though these have also been shown to have their limitations (Austerlitz *et al.* 2004; Robledo-Amuncio *et al.* 2006).

Furthermore, the TWOGENER estimates of pollen dispersal distances rely on an estimate of the *effective* density of pollen donors. It is easy to estimate the density of known adults in a study population by simply counting the number of stems per hectare. However, not all individuals may be reproductively active (available for mating) at precisely the same time and therefore an estimate of the effective density of adults may be markedly different to the number of individuals in the landscape. To quantify the effective adult density requires surveying the overlap in timing of pollen availability and stigma receptivity for a large sample of trees across a study site, which may be a difficult and costly exercise. In this study I modelled the estimate of pollen dispersal distances for declining effective adult density, which revealed that in both species a 10-fold decline in the effective adult density led to a 50% increase in the estimated average distance of pollen dispersal. Furthermore, in species with animal-mediated pollen dispersal, the peculiarities of pollinator foraging behaviour (e.g. changes in response to changing resource availability during the flowering season) or the pattern of pollen

carryover (e.g. if pollen carryover is high, pollen may be dispersed over a greater number of trees than when carryover is low) may further modify the distances that pollen is dispersed. Recent work by Robledo-Arnuncio *et al.* (2006) attempts to model pollen dispersal independently of estimates of effective density, but the authors concede that it remains a difficult task to accurately estimate pollen dispersal using indirect methods.

As discussed in the literature (Smouse *et al.* 2001; Burczyk & Koralewski 2005), paternity analysis also has its limitations. In particular, it can be difficult and costly to sample all potential parents in a plant population in order to accurately measure all pollen gene flow events. In this study, I failed to assign paternity to all offspring of *E. camaldulensis* and *E. leucoxylon* trees. For *E. camaldulensis* I was unable to determine paternity of 25% of offspring and for *E. leucoxylon,* 60% of offspring. Even though I predicted that pollen dispersal in *E. camaldulensis* would be relatively restricted due to the limited mobility of insect-pollinators, I found that pollen dispersal was occurring over very large distances (up to 700m). On the other hand, I was aware that I had sampled only a small proportion of potential *E. leucoxylon* parents, but the paternity analysis also revealed a number of extremely long distance dispersal events (up to 2.5km). Thus, it may be very difficult to sample the entire set of male parents of either species, especially for *E. leucoxylon.*

7.5 Conclusions

In contrast to theoretical predictions, in this study the analysis of patterns of pollen gene flow revealed extremely high levels of genetic connectivity in paddock tree populations of *E. camaldulensis* and *E. leucoxylon.* Pollen dispersal in the insect-pollinated species *E. camaldulensis* was surprisingly widespread and *E. camaldulensis* trees were mating with a greater diversity of individuals than the bird-pollinated species, *E. leucoxylon.* As Dick (2001) found for *D. excelsa* paddock trees, the presence of introduced honeybees may indeed be driving these patterns of long distance pollen dispersal in *E. camaldulensis,* either due to their greater flight capabilities or pollen mixing in the nest. While introduced honeybees are also frequent floral visitors of *E. leucoxylon,* the differences in observed patterns of pollen dispersal may be attributable to the activities of bird pollinators. In particular, it seems that birds may facilitate the extreme long-distance pollen dispersal events in *E. leucoxylon.*

Despite the dominance of eucalypt species in Australian vegetation systems very little is known of the patterns of pollen dispersal in natural populations. Barbour *et al.* (2005) suggest that the majority of pollen dispersal in natural populations occurs within 100m but that low levels of pollen dispersal may occur up to ~1.5km. Indeed, this is the first study of the patterns of pollen dispersal in highly disturbed eucalypt populations, and paternity analyses revealed average distances of pollen dispersal of a magnitude almost three times that estimated by Barbour *et al.* (2005) and ten times that detected in high density eucalypt seed orchards (Chaix *et al.* 2003). Results from the analysis of mating and pollen dispersal patterns amongst *E. camaldulensis* and *E. leucoxylon* paddock trees suggest that both eucalypt species have a reasonably robust mating system (i.e. were able to maintain relatively high outcrossing rates and high levels of pollen dispersal), and, thus, have been more-or-less resilient to the human-induced habitat modifications experienced in the agricultural environment. While female reproductive success is effectively zero under the current grazing regime in the paddock tree environment, these results suggest that paddock trees could certainly contribute to landscape levels of gene flow, at least through male function.

Chapter 8   General discussion and concluding remarks

8.1   General discussion

Habitat fragmentation studies view the landscape as made up of population fragments in a sea of hostile environments (the "matrix", which may be agricultural land, plantation forests, urban habitats etc). Intuitively, we predict that many of the normal ecological properties of intact biological communities are absent in the matrix; however, the ecological properties of the matrix are rarely studied (Kupfer *et al.* 2006). The conversion of natural vegetation systems to agricultural land represents an enormous shift in habitat composition, from a complex ecosystem to one of only a few species, and is predicted to lead to perturbations in many ecological processes, including plant-pollinator mutualisms (Aizen & Feinsinger 1994b; Hobbs & Yates 2003). I investigated the functionality of pollination systems of paddock trees of two temperate woodland eucalypt species to determine the impacts of anthropogenic habitat clearance and tree thinning on plant reproduction. My study revealed that paddock trees, far from being the "living dead" (Janzen 1986), are reproductively viable and may indeed be contributing to substantial landscape-scale connectivity as the result of extensive pollen-mediated gene flow. The significance of paddock trees for regional biodiversity conservation (reviewed in Chapter 1) and the potential contribution of remnant trees to the genetic conservation of each species means that paddock trees are important resources that should be the focus of conservation management plans to ensure the long-term survival and viability of these populations. An understanding of the reproductive ecology of paddock trees will certainly help to guide management decisions.

8.1.1   Review of key findings

As has been found for other paddock tree populations (Carruthers *et al.* 2004), the populations of *E. camaldulensis* and *E. leucoxylon* paddock trees included in my study were highly skewed towards large sized, and most-likely, older-aged individuals. I also found that there was a complete lack of seedling and juvenile individuals in the agricultural paddocks where these trees occurred. Thus, it is likely that these large paddock trees are remnants of the original extant vegetation and the skewed population age structure likely reflects the complete lack of recruitment in the region over the past

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one hundred or so years. The lack of recruitment and the ageing nature of paddock tree populations are of great concern, especially as I found that there was potentially an age-related decline in plant fecundity for both species (Chapter 5). Several authors have estimated the rate of decline in paddock tree numbers to be ~0.5-2.5% a year (Sullivan & Venning 1982; Ozolins *et al.* 2001) and this is likely to increase exponentially as the current adult trees age and senesce. In addition to the ecological and social importance of paddock trees, paddock trees also represent an important store of genetic variation (at least, as indicated by neutral genetic markers; Chapter 4) and have the potential to significantly contribute to the genetic integrity of each species as a whole. Clearly, there is an urgent need for land managers to address the issue of plant regeneration and to halt the rate of paddock tree decline in order to maintain the viability of these populations. An understanding of the reproductive patterns of paddock trees will help to assess the suitability of paddock trees to contribute to regeneration efforts.

As a first step, I surveyed flowering and fruiting patterns of *E. camaldulensis* and *E. leucoxylon* paddock trees in order to assess their reproductive viability. I found that all of the surveyed paddock trees of both species produced flowers and, with the exception of a small number of trees of both species, that the great majority also produced fruits. In addition, seeds produced by paddock trees were viable, as germination rates were also generally high. On a coarse scale this indicates that these paddock trees are of sufficient health to produce flowers (the unit of attraction for pollinators) and to successfully mature fruits containing viable seeds. In addition, for the majority of measures of reproductive output for both species, I found that there were no significant differences in reproductive output between paddock trees and trees found in intact vegetation, again indicating on a coarse scale that paddock tree populations are in good reproductive health.

However, my study was conducted only across two flowering seasons for each species and it is likely that reproductive parameters may vary significantly over longer time frames. For example, reproductive output and mating patterns appeared to vary in *E. camaldulensis* paddock trees across the two flowering seasons and in one season, *E. camaldulensis* trees failed to flower. This variability in reproductive patterns between years is likely to be environmentally driven and the resource allocation of trees to fruits in one year may affect the ability of trees to allocate resources to flowering in the

following year, which has implications for pollinator behaviour. In the years in which my study was conducted, the Mt Lofty Ranges received average yearly rainfall, whereas the region has been experiencing strong drought conditions for several years now. It would be necessary to monitor flowering and fruiting in paddock tree populations over much longer time frames to gain an understanding of the life-time patterns of reproduction in these trees.

I also attempted to determine some of the key tree demographic variables (e.g. tree size, tree isolation, local tree density) that may contribute to the reproductive effort or the female reproductive success of paddock trees. However, I found that there was very little correlation with demographic variables and measures of flower, fruit or seed production. In particular, there was no correlation of measures of fruit and seed production with the distance to nearest conspecific, one of the key factors that I had predicted would have been a major determinant of the likelihood of outcrossed pollination and, therefore, female reproductive success. Surprisingly, even the most spatially isolated trees in my study produced viable seeds and genetic analyses indicated that these seeds were largely outcrossed. This suggests that factors other than the spatial distribution of trees may impact on the reproductive viability of individual trees.

In all cases though, there was great variation in flower, fruit and seed production between individual trees of both species. A number of authors have noted significant variation amongst individuals of eucalypt species in surveys of outcrossing rates in natural populations (e.g. Brown *et al.* 1975; Peters *et al.* 1990; House & Bell 1996; McDonald *et al.* 2003; Butcher *et al.* 2005) so potentially inter-individual variation is a feature of eucalypt reproduction and is, thus, partly genetically determined. However, as each individual paddock tree may be exposed to any of a wide range of modified environmental conditions in the agricultural environment that differ from those under natural conditions (e.g. saline soils, wind exposure, differing distance to nearest mating partners, different densities of mating partners, reduced competition for resources), I expect that a substantial proportion of this reproductive variation in paddock trees is also environmentally determined. I would suggest that paddock trees might experience greater variability in reproductive output than those in natural vegetation, though a more detailed comparative study would be required to determine whether this is the case.

Since *E. camaldulensis* and *E. leucoxylon*, like most eucalypt species, are self-compatible and therefore able to produce seed by self-pollination, it is impossible to determine the genetic "quality" of seeds (e.g. whether seeds are inbred or outbred) collected from these trees without measuring the genetic composition of offspring cohorts. Knowledge of the genetic composition of seedling cohorts may allow us to make predictions regarding the suitability of seed collected from paddock trees for natural regeneration or revegetation projects. For example, seeds produced by selfing may have reduced fitness and/or survival due to the effects of inbreeding depression, which has been shown to occur in a range of eucalypt species (Hardner & Potts 1995a; Hardner & Potts 1997). Furthermore, by examining the patterns of outcrossed paternity it is possible to get an estimate of the number of males contributing to the seed crop and therefore how representative the offspring are of the surrounding adult population.

I provided a snapshot of the mating patterns of a sample of *E. camaldulensis* and *E. leucoxylon* paddock trees across two reproductive seasons. Compared to trees found in natural vegetation, *E. camaldulensis* paddock trees at Tungkillo had approximately a 5% lower outcrossing rate as a result of increased geitonogamous selfing. In particular, *E. camaldulensis* trees found at low density (<1 tree/ha) were the most severely affected, with an outcrossing rate 15% lower than for trees in natural vegetation. Similarly, *E. leucoxylon* paddock trees experienced a 5% decline in outcrossing rate compared to trees in natural vegetation and again, low density *E. leucoxylon* trees had an outcrossing rate 10% lower than trees in natural vegetation. While my study showed that the offspring of paddock trees may contain more selfed individuals than those in natural vegetation, the outcrossing rates of paddock tree populations of both species remained generally high (*E. camaldulensis* 74%; *E. leucoxylon* 82%).

Again, I found few statistically significant correlations between individual outcrossing rates and demographic variables, most likely due to the large variation in individual outcrossing rates. However, I did detect a trend for outcrossing rate to decline with increasing spatial isolation, though this was not clear-cut as even some very isolated trees had high outcrossing rates. On the one hand, it is reassuring that spatially-isolated paddock trees are indeed capable of attracting pollinators and setting outcrossed seed. On the other hand, the observation that selfing increases in low density trees suggests that the situation could only worsen with the continued loss of paddock trees from each

population. Currently the average paddock tree density at both study sites is approximately 1 tree/ha and the average distance to nearest conspecific is ~70m, so it will not be too much longer until plant density declines to <1 tree/ha for the majority of individuals and the degree of spatial isolation increases, perhaps beyond some threshold level leading to reproductive failure (i.e. an Allee effect) (Lamont *et al.* 1993).

Eucalyptus camaldulensis and *E. leucoxylon* are very similar species in their ecological requirements and mode of reproduction, however they differ in their reliance on particular pollinator guilds. *Eucalyptus camaldulensis* is pollinated primarily by insects, while *E. leucoxylon* is pollinated predominantly by birds. I predicted originally that *E. camaldulensis* paddock trees might experience a greater reproductive decline in response to spatial dispersion than *E. leucoxylon* due to the limited mobility of insect pollinators. Indeed, several authors have suggested that distances of 100-250m may effectively isolate some insect-pollinated eucalypt species (Prober & Brown 1994; Butcher *et al.* 2005). Most surprisingly though my study showed that *E. camaldulensis* paddock trees did not suffer a catastrophic decline in reproduction and that, in fact, mating patterns were very similar to that of the bird-pollinated *E. leucoxylon*. While *E. camaldulensis* trees experienced higher levels of selfing than *E. leucoxylon*, the patterns of outcrossing and pollen dispersal in *E. camaldulensis* paddock trees were remarkably similar to that of *E. leucoxylon*. Overall, *E. camaldulensis* paddock trees tended to mate with a greater number of individuals than *E. leucoxylon* paddock trees and paternity analysis showed that *E. camaldulensis* trees were less frequently mating with their nearest neighbours than *E. leucoxylon* paddock trees. Though sample sizes were limited, mating system analyses suggested that low density *E. camaldulensis* paddock trees (the most spatially isolated) mated with twice the number of individuals that low density *E. leucoxylon* paddock trees did. However, it would be necessary to measure the degree of phenological overlap in trees of each species to determine their "effective" density in the landscape, which would provide insight into whether these patterns were driven by pollinator behaviour or simply by the spatial distribution of available mating partners.

As suggested by mating system analysis, surprisingly high levels of pollen dispersal were detected in the paternity analysis performed on a subset of *E. camaldulensis* paddock trees. For the outcrossed seedlings for which paternity could be assigned to an

individual within the study area, the average distance of outcrossed pollen dispersal was 300m, three times greater than the average nearest neighbour distance within the sampled trees. Since there were also a number of seedlings for which paternity could not be assigned to trees within the study area, this could be considered a minimum estimate of the average distance of pollen dispersal in *E. camaldulensis* paddock trees. Paternity analysis of *E. leucoxylon* seedlings also revealed very high levels of connectivity between remnant trees and due to the difference in sampling design between the two species, I was able to detect significant levels of very long-distance pollen dispersal (up to 2.3km) in *E. leucoxylon* trees. While there were a number of very long-distance mating events detected in *E. leucoxylon* paddock trees, the median distance of outcrossed pollen dispersal was 184m in this species. In addition, the paternity analysis of this species revealed that there was high connectivity between trees in remnant patches (high density trees) and more isolated paddock trees, highlighting the important role that paddock trees may play in providing connectivity between isolated patches of vegetation and in extending the pollination neighbourhoods of trees in remnant patches.

I found that the agricultural paddock tree environment at my two study sites provided sufficient habitat and resources for the maintenance of pollination services for the remnant *E. camaldulensis* and *E. leucoxylon* trees that remain. However, caution should be applied when generalising these results to other locations or to other eucalypt species for a number of reasons. Firstly, the majority of trees that I surveyed in this study were in reasonable to good health and did not visibly appear to suffer from dieback or other severe health problems. Although not measured, I also assume that nectar production in the paddock trees surveyed was sufficient to sustain pollinator populations as most trees received adequate outcrossed pollination. This may certainly not be the case for other paddock tree populations as in many areas throughout southern Australia paddock trees are in poor health due to dieback, lerp infestation, rising salinity or soil acidification, amongst other problems (Reid & Landsberg 1999). The poor health of other paddock tree species may interfere with their ability to produce flowers and nectar and therefore to provide a sufficient reward to encourage pollinator movements across the large spatial distances that separate trees.

As indicated by the mating system results, invertebrate pollinator movement patterns were sufficient to provide adequate pollination services to *E. camaldulensis* trees located up to 300m distant to other individuals, in contrast to what has been reported for other insect-pollinated eucalypt species (Prober & Brown 1994; Butcher *et al.* 2005). While *E. camaldulensis* is visited by a suite of native invertebrate fauna, including bees, wasps and flies, *E. camaldulensis* is also very heavily visited by the introduced European honeybee, *Apis mellifera*. These large bees are capable of flight distances of up to 9km (Beekman & Ratnieks 2000) but most frequently make foraging bouts of up to ~1km (Waddington *et al.* 1994). *Eucalyptus camaldulensis* is used for honey production in many regions of South Australia (Paton *et al.* 2004a) as it flowers frequently and produces numerous, nectar-rich flowers. *Eucalyptus camaldulensis* paddock trees, therefore, are likely to represent a predictable and abundant food resource to sustain honeybee populations. In addition, the reward represented by individual trees may be sufficient to overcome the opportunity-cost for honeybees to move between spatially isolated trees, facilitating high levels of pollen dispersal amongst paddock trees. This may not be the case for other smaller insect pollinator species or for other eucalypt species that produce a lesser nectar or pollen reward than *E. camaldulensis*.

Lastly, *E. camaldulensis* and *E. leucoxylon* are both widely-distributed species. *Eucalyptus camaldulensis* has a distribution throughout much of northern and south-eastern Australia, while *E. leucoxylon* is distributed across the south-eastern portion of Australia from Victoria through to the Nullarbor Plain. Such an extensive distribution for each species suggests that each may be tolerant of a wide range of environmental and ecological conditions, and the results from my study suggest that both species have been mostly resilient to the serious perturbations brought about by habitat clearance and vegetation thinning. It is possible that other eucalypt species with a more restricted distribution may be less tolerant of human-mediated habitat changes, perhaps due to a narrower ecological range, reliance on more specialised pollinators, limited dispersal potential or any range of factors that has limited the distribution of the species in the first place.

8.1.2 Implications for paddock tree regeneration

Genetic analyses of the mating systems of *E. camaldulensis* and *E. leucoxylon* trees suggest that seed from paddock trees may be suitably genetically diverse to contribute to the long term regeneration of paddock tree populations. In both species, seedling cohorts were genetically representative of adult populations in terms of the number of microsatellite alleles detected and allelic frequencies. In addition, individual mating system analyses indicated that the offspring of paddock trees capture a significant proportion of the genetic diversity of surrounding adult trees as adults mate with a wide range of individuals over a large spatial scale (up to 2.5km). Ideally, in order to preserve "locally" adapted gene complexes and to reduce the risk of genetic pollution from non-local seed sources, it would be best practice to use seed collected *in situ* from paddock trees in regeneration or revegetation projects as paddock trees are often the last remnants of the pre-existing vegetation in any one area. Analysis of individual mating patterns suggest that the optimal strategy would be to collect seed or allow natural regeneration from paddock trees found in medium to high density arrays, as low density trees of both species had a higher degree of selfing than these trees. In addition, trees found in medium to high density situations were found to be mating with a greater number of individuals and therefore are capturing a greater proportion of the surrounding genetic variation. Further to this, it would be best practice to collect seed for revegetation projects from throughout the canopy of paddock trees as this study showed outcrossing rates may vary across the canopy, with seeds high in the canopy more likely to be outcrossed.

Nonetheless, low density paddock trees may still make a valuable contribution to regeneration as many still had moderately high outcrossing rates, and especially for *E. camaldulensis*, were still mating with a large number of individuals. While these trees had a higher level of selfing, it is currently unknown the degree to which either *E. camaldulensis* or *E. leucoxylon* may suffer inbreeding depression. Genetic surveys of the adult tree populations of each species revealed paddock trees were highly heterozygous and that overall, paddock trees were genetically unrelated (i.e. no full or half-sibs were detected in the adult populations). Greater observed than expected heterozygosity in adult trees suggests that there is indeed strong selection against homozygous (potentially selfed) individuals. However, vegetation clearance may also

have had the effect of removing related individuals (selfed individuals as well as sibs) from the population, since clearing all but single spatially-separated trees would have removed any family structure that normally occurs in eucalypt populations due to limited seed dispersal (Skabo *et al.* 1998), which would have contributed to the high levels of heterozygosity detected in the population. Further experiments would be required to determine the performance of selfed individuals in the paddock tree environment and whether they do suffer a loss of fitness and/or reduced survival as suggested for other eucalypt species. The regeneration of moderate levels of selfed progeny may though provide some level of "reproductive assurance" for paddock trees and slow the erosion of genetic diversity that is occurring with the continued loss of individual trees.

In this study I measured the genetic composition of offspring cohorts at the point three weeks post-germination. Genetic analyses suggested that offspring cohorts of paddock trees at this life stage are highly outcrossed and genetically diverse and would notionally be suitable for plant regeneration. However, I did not measure seedling survival past more than a few weeks and the only published study to measure the survival of progeny from paddock trees (Rocha & Aguilar 2001) suggests that studies that include much longer time frames are needed to adequately assess the suitability of seed collected from paddock trees to contribute to population regeneration. Rocha and Aguilar (2001) found no difference in the outcrossing rate of seeds collected from *Enterolobium cyclocarpum* paddock trees and seed collected from trees in continuous vegetation (t_m=0.99 and 1.0, respectively), but they found that seedling survival and growth of progeny from paddock trees was much lower than that from trees in continuous vegetation. This suggests that *E. cyclocarpum* paddock trees may be making incompatible matings, potentially as a result of the reduction of the number of available mating partners. In addition, with the large average distance of pollen gene flow detected in some remnant tree populations (e.g. White *et al.* 2002; Dick *et al.* 2003; this study), the progeny of paddock trees may also be susceptible to outbreeding depression – the decline in offspring fitness caused by genetic incompatibility and the breakdown of locally adapted gene complexes. Hardner *et al.* (1998) found no effect of pollination distance (500m to 100km) on early seedling growth and survival of *E. globulus* in a common garden experiment, suggesting the absence of outbreeding depression in eucalypts. However, further experiments would be required to determine

whether outbreeding depression may be manifested at later stages or whether the effects are greater under natural field conditions. Since pollen dispersal distances detected in *E. camaldulensis* and *E. leucoxylon* paddock trees were much greater than predicted, there is a risk that the offspring of paddock trees of these species may also be susceptible to some level of outbreeding depression.

Clearly, it would be necessary to investigate whether the type of matings that I have documented occurring in paddock trees have produced offspring with high levels of survival and fitness in the paddock tree environment. Potentially, the progeny of paddock trees may exhibit inbreeding depression, as a result of increased selfing, or outbreeding depression, as a result of incompatible matings due to long-distance pollen flow. However, a greater level of gene dispersal as detected in these trees may in fact provide a means for the long term persistence of paddock tree populations, especially in the face of ongoing modification and degradation of agricultural environments and with global climate change predicted to significantly alter climactic patterns in southern Australia (Hughes 2003). The mixing of genes over a greater spatial area may provide for novel genotypes that have higher fitness under new environmental regimes and/or facilitate the colonisation of new habitat. Collecting seed from paddock trees for use in *in situ* revegetation projects has the effect of preserving some of the genes that may be important in local-adaptation, while long-distance pollen dispersal has the effect of introducing novel genes that may be beneficial under changing environmental regimes.

8.2 Directions for future research

My study provided a snapshot of the reproductive and mating patterns of *E. camaldulensis* and *E. leucoxylon* paddock trees in one population of each species over two flowering seasons. The study was intended as an exploration of some of the demographic and ecological factors that may influence mating patterns at a single location for each species, since all trees are likely to be subject to the same pollination conditions at the one site. Indeed my study provided some interesting insights into how tree demography may influence mating patterns but it is impossible to make statements about the generality of these findings for each species without first extending this study to other populations and to other species. In particular, I think it would be interesting to replicate this study across a much broader area, but within the same region (to ensure

trees are likely to experience similar pollination and environmental conditions). I found that the distance to nearest neighbour was not a good predictor of outcrossing rate for individual trees but that clearer patterns were observed when comparing trees with different densities of potential mating partners. Consequently, I would ensure that there was greater replication of trees of each density category (e.g. low, medium and high density paddock trees) to confirm that some of the observations of my study were indeed representative and not just due to local conditions.

One of the most surprising outcomes of my study was the high level of pollen-mediated gene flow amongst *E. camaldulensis* paddock trees, a species with predominant insect pollination. Potentially these patterns may have been driven by the activities of the introduced honeybee and it would be particularly interesting to replicate this study in other eucalypt species not heavily visited by honeybees. In addition, as identified above, it would be interesting to choose species in which floral and nectar production are lesser than that in *E. camaldulensis* and *E. leucoxylon* to assess whether the resource value of individual paddock trees influences spatial patterns of pollinator foraging.

I found that the genetic composition of seedling cohorts from paddock trees varied significantly between the two flowering seasons for each species. While the summary measures of genetic diversity and mating system parameters suggest that the overall genetic outcome is essentially the same between years, the finer-scale detail of the mating system analyses indicates that individual mating patterns may vary between years. Variation between years may be driven by environmental conditions, for example, drought versus heavy rainfall years may influence the patterns of flowering and/or the abundance of pollinators leading to changes in the number of available mating partners or pollinator foraging behaviour. Replicating this study across multiple flowering seasons would allow us to gain an understanding of how mating patterns may change over time and how they are influenced by particular conditions. Replicating this study across a larger time-frame would perhaps also provide insights into the potential life-time reproductive output of paddock trees.

Many animals use paddock trees as "stepping stones" for movement across the landscape. Analysis of the patterns of pollen dispersal in paddock trees confirmed that pollinators are also making large movements across the landscape and, in addition, are

moving between patches of remnant vegetation and paddock trees. In this way, paddock trees may indeed extend the genetic neighbourhoods of trees in remnant patches and provide genetic connectivity between patches of vegetation across the landscape, facilitating species conservation across a much larger spatial scale. To determine the degree of connectivity between remnant vegetation patches and paddock tree populations it would be useful to use a similar genetic approach as to the one I have used here. Paternity analysis could be used to determine the extent of pollen immigration and/or emigration to and from remnant patches of vegetation to paddock trees and the spatial scale over which this occurs. For effective gene flow to occur though, offspring need to survive to reproductive maturity, and in the paddock tree environment this is unlikely to occur. Thus, at this stage, gene flow is likely to be unidirectional – into remnant patches of vegetation protected from grazing.

Mating system analyses identified some interesting aspects of the response of different pollinators to changes in the spatial structure of plant populations. In particular, genetic analysis suggested that bird and insect pollinator behaviour differed in response to the clumping of paddock trees. In *E. leucoxylon*, high density trees had a lower outcrossing rate but received pollen from a greater range of individuals than other trees. In contrast, *E. camaldulensis* trees in high density patches had a much higher outcrossing rate than other trees but received pollen only from a few individuals. This suggests that bird pollinators may be visiting a large number of individuals in high density patches, whereas insect-pollinators may only visit a few individuals (assuming that the rate of pollen carryover is the same between each species). Furthermore, the paternity analyses indicated that low density paddock trees were not necessarily mating with their nearest neighbours. This seems counterintuitive considering the opportunity-costs for pollinators associated with travelling large distances between trees. To confirm the difference in pollinator responses suggested by these analyses it would be interesting to examine the foraging behaviour of bird and insect pollinators on scattered trees and their patterns of movement between trees, especially to gain an understanding of the foraging decisions pollinators may make. It would be ideal to use satellite or radio-tracking technology to achieve this, considering the large spatial scale over which pollinators appear to be foraging, however, such technology is not currently available for use with such small species. An additional indirect approach would be to refine DNA and genetic assignment methods to genotype the pollen collected from foraging

animals and to assign the pollen to the source tree. In this way we could quantify the proportional representation of particular individuals carried in the pollen load and gain an understanding of the number of trees visited in a foraging bout and the rate of pollen carryover.

8.3 Concluding remarks

Evidence from habitat fragmentation studies suggest that many plant species suffer reproductive decline due to alterations in mating patterns brought about by the decline of pollinator species or changes in pollinator behaviour in response to habitat loss (Hobbs & Yates 2003; Aguilar *et al.* 2006). For paddock trees, the single trees that are left in the agricultural environment following habitat clearance and tree thinning, these effects could be predicted to be especially severe due to the drastic alteration in the spatial configuration of populations and the loss of a diverse natural habitat. This study however contributes to the growing body of evidence that suggests that some plant species may in fact be resilient to the significant perturbations caused by conversion of natural habitat to agricultural land. *Eucalyptus camaldulensis* and *E. leucoxylon* paddock trees included in this study were reproductively viable and, on the whole, produced genetically diverse offspring that were genetically representative of the adult populations. As such, it seems a misnomer to describe paddock trees as "isolated trees" as there was substantial connectivity across the study populations of both *E. camaldulensis* and *E. leucoxylon* paddock trees. As has been found for a number of other species (e.g. Chase *et al.* 1996; White *et al.* 2002; Dick *et al.* 2003), pollen dispersal in both *E. camaldulensis* and *E. leucoxylon* paddock tree populations was extensive and highlights the importance of paddock trees in facilitating landscape-level gene flow. However, while genetic analyses suggested that seed collected from these paddock trees may be suitable for plant regeneration, further experiments are required to assess the long-term survival and fitness of offspring of paddock trees, and hence, their potential contribution to the long-term viability of paddock tree populations.

Appendix 1: Location of voucher specimens of eucalypt species in which the conservation of *E. leucoxylon* microsatellite primers were tested.

Species	Voucher Reference	Specimen Location[a]
E. leucoxylon	El5602	Kebbles, Flaxley
E. leucoxylon pruinosa	Elpr1	Row 45 Tree 10, CCA
E. leucoxylon pruinosa	Elpr2	Row 45,Tree 11, CCA
E. petiolaris	Epet1	Row 74 Tree 29, CCA
E. petiolaris	Epet2	G844111, ABG
E. melliodora	Emel1	Row 45 Tree 13, CCA
E. melliodora	Emel2	Tree 1576, WA
E. camaldulensis	Ec3278	Roskhill, Tungkillo
E. camaldulensis	Ec3346	Guthries, Tungkillo
E. fasiculosa	Efas1	Tree 1206, WA
E. fasiculosa	Efas2	W861670, ABG
E. tetraptera	Etet1	Row 42 tree 29, CCA
E. tetraptera	Etet2	Tree 1860b, WA
E. salmonophloia	Esal1	Row 42 Tree 14, CCA
E. salmonophloia	Esal2	Tree 47, WA
E. viminalis (ssp cygnetensis)	Evim1	Tree 88b, WA
E. viminalis	Evim2	Row 146 Tree 21, CCA
E. microcarpa	Emicp1	Blackwood
E. microcarpa	Emicp2	Belair National Park
E. grandis	Egr1	Row 165 Tree 26, CCA
E. grandis	Egr2	Row 93 Tree 35, CCA
E. sieberi	Esie1	Row 141 Tree 21, CCA
E. sieberi	Esie2	Row 141 Tree 22, CCA
E. obliqua	Eob1	Tree 46, WA
E. obliqua	Eob2	Row 72 Tree 38, CCA
E. marginata	Emar1	Row 72 Tree 26, CCA
E. marginata	Emar2	Row 26 Tree 29, CCA
E. cloeziana	Ecl1	Row 56 Tree 23, CCA
E. cloeziana	Ecl2	Tree 1620, WA
E. royceii	Eroy1	Row 63 Tree 22, CCA
E. royceii	Eroy2	Row 63 Tree 22, CCA
E. eudesmoides	Eeu1	Row 25 Tree 34, CCA
E. eudesmoides	Eeu2	Row 25 Tree 37, CCA
E. baileyana	Eba1	Row 52 Tree 21, CCA
Angophora costata	Acos1	Row 158 Tree 9, CCA
Angophora costata	Acos2	Tree 51, WA
Corymbia calophylla	Ccal1	Row 72 Tree 27, CCA
Corymbia calophylla	Ccal2	Tree 154, WA

[a] ABG – Adelaide Botanic Gardens; CCA – Currency Creek Arboretum ; WA – Waite Arboretum.

Appendix 2: Allele frequencies of *E. camaldulensis* and *E. leucoxylon* adults and seedling cohorts

a. *E. camaldulensis*

Locus	Allele	Adults	Seedlings 2000	Seedlings 2002
Eg99	181	0.000	0.000	0.006
	187	0.073	0.067	0.063
	191	0.271	0.260	0.286
	194	0.385	0.387	0.409
	197	0.250	0.282	0.209
	200	0.021	0.004	0.020
	203	0.000	0.000	0.006
Eg98	166	0.135	0.121	0.110
	169	0.042	0.040	0.014
	172	0.271	0.255	0.324
	175	0.406	0.430	0.393
	178	0.115	0.119	0.119
	181	0.021	0.030	0.031
	184	0.010	0.002	0.009
	190	0.000	0.002	0.000
Eg16	234	0.000	0.008	0.005
	236	0.323	0.362	0.255
	237	0.281	0.259	0.325
	240	0.365	0.337	0.373
	243	0.021	0.035	0.030
	244	0.000	0.000	0.005
	246	0.010	0.000	0.008
Eg91	130	0.000	0.000	0.002
	134	0.043	0.002	0.034
	136	0.138	0.126	0.120
	138	0.021	0.017	0.034
	140	0.298	0.378	0.270
	142	0.064	0.059	0.079
	145	0.362	0.349	0.391
	148	0.043	0.055	0.026
	151	0.032	0.010	0.039
	154	0.000	0.002	0.004
	157	0.000	0.002	0.000
Eg65	231	0.000	0.008	0.000
	234	0.542	0.542	0.571
	237	0.010	0.027	0.005
	240	0.010	0.008	0.012
	243	0.135	0.121	0.104
	246	0.125	0.123	0.167
	249	0.073	0.061	0.056
	255	0.042	0.052	0.031
	258	0.010	0.015	0.018
	261	0.010	0.015	0.018
	267	0.010	0.006	0.000

Locus	Allele	Adults	Seedlings 2000	Seedlings 2002
	270	0.031	0.021	0.017
	273	0.000	0.002	0.000
Eg96	274	0.083	0.120	0.137
	277	0.042	0.040	0.039
	280	0.573	0.605	0.465
	283	0.208	0.156	0.266
	286	0.031	0.019	0.044
	289	0.021	0.021	0.020
	292	0.042	0.038	0.030
Eg84	101	0.122	0.080	0.126
	104	0.167	0.203	0.198
	107	0.122	0.091	0.144
	110	0.144	0.174	0.106
	113	0.067	0.053	0.090
	116	0.144	0.188	0.129
	119	0.056	0.049	0.034
	122	0.022	0.023	0.015
	125	0.033	0.019	0.054
	128	0.111	0.100	0.093
	131	0.011	0.021	0.010

b. *E. leucoxylon*

Locus	Allele	Adults	Seedlings 2001	Seedlings 2003
EL14	164	0.000	0.000	0.002
	166	0.179	0.160	0.143
	168	0.161	0.127	0.131
	170	0.036	0.058	0.028
	172	0.000	0.032	0.009
	174	0.018	0.002	0.030
	176	0.071	0.063	0.049
	178	0.036	0.038	0.040
	180	0.179	0.177	0.150
	182	0.036	0.047	0.058
	184	0.125	0.117	0.107
	186	0.000	0.010	0.009
	188	0.000	0.007	0.005
	190	0.071	0.078	0.091
	192	0.089	0.078	0.140
	194	0.000	0.005	0.005
	196	0.000	0.000	0.002
EL01	350	0.000	0.000	0.003
	358	0.339	0.218	0.338
	360	0.018	0.006	0.005
	362	0.018	0.003	0.003
	364	0.000	0.003	0.000
	366	0.018	0.026	0.021
	374	0.571	0.695	0.592
	386	0.036	0.049	0.038
EL07	117	0.000	0.008	0.000

Locus	Allele	Adults	Seedlings 2001	Seedlings 2003
	119	0.179	0.135	0.142
	121	0.143	0.152	0.108
	123	0.161	0.155	0.210
	125	0.161	0.178	0.179
	127	0.179	0.157	0.156
	129	0.036	0.069	0.066
	131	0.000	0.003	0.005
	133	0.000	0.003	0.009
	135	0.018	0.000	0.007
	137	0.018	0.018	0.012
	139	0.000	0.010	0.005
	141	0.036	0.036	0.035
	143	0.000	0.008	0.000
	145	0.036	0.020	0.040
	147	0.000	0.008	0.000
	149	0.000	0.005	0.000
	151	0.036	0.038	0.021
	161	0.000	0.000	0.005
EL28	188	0.000	0.005	0.000
	191	0.036	0.029	0.017
	203	0.000	0.000	0.002
	206	0.375	0.318	0.391
	209	0.018	0.058	0.052
	212	0.000	0.007	0.005
	215	0.089	0.141	0.111
	218	0.018	0.058	0.036
	221	0.464	0.383	0.386
EL18	279	0.019	0.016	0.002
	281	0.000	0.005	0.000
	283	0.019	0.000	0.002
	287	0.111	0.088	0.108
	289	0.000	0.008	0.005
	291	0.037	0.037	0.034
	293	0.093	0.067	0.089
	295	0.185	0.136	0.111
	297	0.148	0.171	0.156
	299	0.093	0.152	0.125
	301	0.019	0.021	0.012
	303	0.019	0.011	0.022
	305	0.000	0.005	0.005
	307	0.037	0.056	0.055
	309	0.093	0.107	0.108
	311	0.056	0.029	0.055
	313	0.056	0.061	0.079
	315	0.000	0.003	0.014
	317	0.000	0.003	0.007
	321	0.000	0.003	0.000
	323	0.000	0.003	0.000
	325	0.019	0.016	0.010
EL13	172	0.036	0.022	0.002
	174	0.000	0.005	0.002
	176	0.000	0.010	0.002
	182	0.054	0.043	0.059

Locus	Allele	Adults	Seedlings 2001	Seedlings 2003
	184	0.000	0.007	0.000
	186	0.054	0.026	0.033
	188	0.054	0.031	0.059
	190	0.143	0.139	0.162
	192	0.018	0.031	0.040
	196	0.000	0.010	0.005
	198	0.107	0.113	0.087
	200	0.375	0.406	0.408
	202	0.143	0.125	0.136
	204	0.018	0.026	0.005
	206	0.000	0.002	0.000
	208	0.000	0.002	0.000
EL16	222	0.036	0.023	0.022
	228	0.036	0.023	0.030
	230	0.089	0.107	0.054
	232	0.500	0.513	0.540
	234	0.071	0.071	0.072
	236	0.089	0.066	0.067
	238	0.054	0.074	0.109
	240	0.054	0.033	0.052
	242	0.018	0.020	0.015
	244	0.018	0.010	0.007
	248	0.036	0.061	0.032
EL29	258	0.018	0.020	0.020
	260	0.036	0.028	0.007
	262	0.000	0.005	0.000
	264	0.000	0.010	0.002
	266	0.250	0.245	0.202
	268	0.286	0.222	0.346
	270	0.125	0.112	0.134
	272	0.161	0.171	0.127
	274	0.107	0.156	0.134
	276	0.018	0.026	0.022
	280	0.000	0.003	0.000
	282	0.000	0.003	0.005

Appendix 3: Family estimates of mating system parameters (± S.E.) of *E. camaldulensis* and *E. leucoxylon* trees
[a] sampled in Year1 only, [b] sampled in Year2 only, [c] sampled in both years (data from both years analysed together).

a. *E. camaldulensis*

Family ID	Tree Density	Distance to Nearest Conspecific (m)	Multilocus outcrossing rate (t_m)	Single-locus outcrossing rate (t_s)	Biparental Inbreeding (t_m-t_s)	Correlation of paternity (r_p)
Ec3276[c]	Natural	10	0.77 ± 0.37	0.60 ± 0.29	0.17 ± 0.08	0.27 ± 0.03
Ec3278[c]	Natural	20	0.56 ± 0.27	0.49 ± 0.23	0.08 ± 0.04	0.12 ± 0.02
Ec3280[c]	Natural	3	1.20 ± 0.57	0.70 ± 0.34	0.51 ± 0.23	0.29 ± 0.04
Ec3301[b]	Low	142	0.75 ± 0.36	0.53 ± 0.26	0.22 ± 0.10	0.05 ± 0.01
Ec3302[c]	Low	148	0.52 ± 0.25	0.44 ± 0.21	0.08 ± 0.04	0.29 ± 0.04
Ec3305[a]	Low	207	0.56 ± 0.27	0.33 ± 0.16	0.12 ± 0.02	0.25 ± 0.04
Ec3317[c]	Low	46	0.32 ± 0.15	0.15 ± 0.07	0.16 ± 0.08	0.74 ± 0.08
Ec3319[c]	Low	67	0.76 ± 0.37	0.65 ± 0.32	0.11 ± 0.05	0.00 ± 0.01
Ec3322[a]	Low	30	0.80 ± 0.38	0.73 ± 0.35	0.07 ± 0.04	0.08 ± 0.01
Ec3323[a]	Low	121	0.50 ± 0.24	0.49 ± 0.24	0.01 ± 0.01	0.00 ± 0.02
Ec3328[b]	Low	142	1.20 ± 0.58	0.82 ± 0.40	0.38 ± 0.18	0.17 ± 0.02
Ec3330[a]	Low	206	0.31 ± 0.15	0.26 ± 0.12	0.05 ± 0.02	0.00 ± 0.02
Ec3332[b]	Low	110	1.20 ± 0.58	0.77 ± 0.38	0.43 ± 0.21	0.29 ± 0.04
Ec3333[b]	Low	20	0.74 ± 0.36	0.55 ± 0.27	0.19 ± 0.09	0.47 ± 0.06
Ec3336[a]	Low	221	0.86 ± 0.41	0.81 ± 0.38	0.05 ± 0.03	0.00 ± 0.04
Ec3337[c]	Low	164	0.90 ± 0.43	0.81 ± 0.39	0.09 ± 0.05	0.02 ± 0.00
Ec3338[c]	Low	220	0.81 ± 0.39	0.70 ± 0.34	0.11 ± 0.06	0.11 ± 0.02
Ec3339[a]	Low	334	0.67 ± 0.32	0.50 ± 0.24	0.17 ± 0.08	0.50 ± 0.08
Ec3341[c]	Low	214	1.20 ± 0.57	0.95 ± 0.45	0.25 ± 0.12	0.00 ± 0.01
Ec3342[a]	Low	267	0.89 ± 0.43	0.62 ± 0.30	0.27 ± 0.13	0.25 ± 0.04
Ec3344[a]	Low	324	0.21 ± 0.10	0.12 ± 0.06	0.09 ± 0.04	0.66 ± 0.10

Ec3345[a]	Low	192	0.07 ± 0.03	0.02 ± 0.01	0.05 ± 0.02	0.00 ± 0.03
Ec3346[c]	Low	61	0.93 ± 0.45	0.83 ± 0.40	0.10 ± 0.05	0.00 ± 0.02
Ec3347[c]	Low	87	0.43 ± 0.21	0.32 ± 0.16	0.11 ± 0.05	0.17 ± 0.02
Ec3349[c]	Low	98	0.99 ± 0.48	0.80 ± 0.39	0.19 ± 0.09	0.05 ± 0.01
Ec3350[a]	Low	41	0.38 ± 0.18	0.20 ± 0.10	0.18 ± 0.08	0.37 ± 0.05
Ec01[b]	Medium	26	0.40 ± 0.20	0.30 ± 0.14	0.11 ± 0.05	0.00 ± 0.04
Ec3282[b]	Medium	46	1.20 ± 0.58	0.75 ± 0.37	0.45 ± 0.22	0.15 ± 0.02
Ec3283[c]	Medium	18	0.54 ± 0.26	0.46 ± 0.22	0.08 ± 0.04	0.03 ± 0.00
Ec3284[b]	Medium	46	1.20 ± 0.57	0.80 ± 0.38	0.41 ± 0.19	0.34 ± 0.04
Ec3290[b]	Medium	45	1.20 ± 0.58	0.66 ± 0.32	0.54 ± 0.26	0.05 ± 0.01
Ec3291[b]	Medium	38	0.56 ± 0.27	0.56 ± 0.27	0.00 ± 0.01	0.00 ± 0.03
Ec3292[b]	Medium	23	0.90 ± 0.44	0.54 ± 0.27	0.36 ± 0.17	0.48 ± 0.07
Ec3294[b]	Medium	15	1.20 ± 0.57	0.89 ± 0.43	0.31 ± 0.15	0.00 ± 0.03
Ec3295[b]	Medium	52	0.90 ± 0.42	0.61 ± 0.29	0.28 ± 0.13	0.31 ± 0.36
Ec3296[b]	Medium	70	0.70 ± 0.33	0.66 ± 0.31	0.05 ± 0.02	0.17 ± 0.03
Ec3297[b]	Medium	49	0.71 ± 0.34	0.59 ± 0.29	0.11 ± 0.06	0.12 ± 0.02
Ec3311[c]	Medium	20	0.70 ± 0.34	0.48 ± 0.23	0.22 ± 0.11	0.17 ± 0.02
Ec3315[c]	Medium	60	0.80 ± 0.38	0.69 ± 0.33	0.11 ± 0.05	0.07 ± 0.01
Ec3348[a]	Medium	32	1.20 ± 0.57	0.72 ± 0.34	0.48 ± 0.22	0.14 ± 0.02
Ec6499[b]	Medium	70	1.20 ± 0.58	0.94 ± 0.45	0.26 ± 0.13	0.05 ± 0.01
Ec3285[c]	High	3	0.66 ± 0.32	0.61 ± 0.29	0.06 ± 0.03	0.25 ± 0.03
Ec3286[b]	High	2	0.64 ± 0.31	0.57 ± 0.28	0.07 ± 0.03	0.00 ± 0.02
Ec3287[c]	High	2	0.85 ± 0.41	0.62 ± 0.30	0.24 ± 0.11	0.27 ± 0.03
Ec3288[b]	High	4	0.71 ± 0.34	0.47 ± 0.22	0.24 ± 0.12	1.00 ± 0.13
Ec3289[c]	High	1	1.20 ± 0.58	0.91 ± 0.44	0.30 ± 0.14	0.02 ± 0.0
Average			**0.78 ± 0.08**	**0.59 ± 0.07**	**0.19 ± 0.06**	**0.19 ± 0.07**
Minimum			**0.07**	**0.02**	**0.0**	**0.0**
Maximum			**1.2**	**0.95**	**0.54**	**1.0**

b. *E. leucoxylon*

Family ID	Density	Distance to nearest conspecific (m)	Multilocus outcrossing rate (t_m)	Singlelocus outcrossing rate (t_s)	Biparental Inbreeding (t_m-t_s)	Multilocus correlation of paternity (r_p)
El 5601[c]	Low	33	0.40 (0.19)	0.31 (0.15)	0.09 (0.05)	0.43 (0.08)
El 5602[b]	Low	33	1.20 (0.58)	0.59 (0.29)	0.61 (0.30)	0.60 (0.10)
El 5606[c]	Low	152	1.20 (0.57)	0.88 (0.42)	0.32 (0.15)	0.04 (0.01)
El 5607[c]	Low	154	1.20 (0.58)	0.79 (0.39)	0.41 (0.19)	0.45 (0.08)
El 5608[a]	Low	150	0.50 (0.24)	0.48 (0.23)	0.02 (0.02)	0.06 (0.01)
El 5609[c]	Medium	35	0.84 (0.41)	0.82 (0.40)	0.02 (0.01)	0.07 (0.01)
El 5610[a]	Medium	5	1.20 (0.57)	0.68 (0.33)	0.52 (0.24)	0.45 (0.08)
El 5611[a]	Medium	5	0.78 (0.37)	0.42 (0.20)	0.36 (0.17)	0.39 (0.07)
El 5612[b]	Medium	10	1.20 (0.58)	0.97 (0.48)	0.23 (0.11)	0.18 (0.03)
El 5613[b]	Low	62	1.20 (0.57)	0.92 (0.44)	0.28 (0.13)	0.17 (0.03)
El 5615[c]	Low	75	0.29 (0.14)	0.29 (0.14)	0.00 (0.01)	0.00 (0.07)
El 5618[c]	High	5	0.89 (0.43)	0.72 (0.35)	0.16 (0.08)	0.14 (0.02)
El 5619[c]	High	5	1.20 (0.56)	0.85 (0.40)	0.35 (0.16)	0.10 (0.02)
El 5620[c]	High	10	0.60 (0.29)	0.50 (0.24)	0.10 (0.05)	0.27 (0.05)
El 5621[c]	High	10	0.35 (0.17)	0.18 (0.08)	0.17 (0.08)	0.36 (0.06)
El 5622[c]	Low	382	1.20 (0.58)	0.92 (0.44)	0.28 (0.14)	0.00 (0.04)
El 5623[c]	Low	60	0.50 (0.24)	0.30 (0.14)	0.20 (0.10)	0.25 (0.05)
El 6451[c]	Low	2	1.20 (0.57)	0.75 (0.36)	0.45 (0.21)	0.53 (0.08)
El 6454[c]	Low	22	1.20 (0.57)	0.73 (0.35)	0.47 (0.22)	0.70 (0.14)
El 6455[c]	Low	35	1.20 (0.58)	0.75 (0.36)	0.45 (0.22)	0.06 (0.01)
El 6457[b]	Medium	20	0.80 (0.38)	0.69 (0.33)	0.11 (0.05)	0.66 (0.18)
El 6458[a]	Medium	10	0.90 (0.44)	0.64 (0.31)	0.26 (0.12)	0.11 (0.02)
El 6461[c]	Natural	4	1.20 (0.57)	0.76 (0.37)	0.44 (0.20)	0.21 (0.04)
El 6462[c]	Natural	5	0.75 (0.36)	0.49 (0.23)	0.26 (0.12)	0.14 (0.02)
El 6463[c]	Natural	3	0.85 (0.41)	0.60 (0.29)	0.25 (0.12)	0.14 (0.02)
El 6492[b]	Low	88	0.90 (0.43)	0.59 (0.28)	0.31 (0.15)	0.50 (0.09)

Family ID	Density	Distance to nearest conspecific (m)	Multilocus outcrossing rate (t_m)	Singlelocus outcrossing rate (t_s)	Biparental Inbreeding $(t_m - t_s)$	Multilocus correlation of paternity (r_p)
El 6493 [a]	Low	32	0.62 (0.29)	0.32 (0.15)	0.30 (0.14)	0.31 (0.06)
Average (SE)			0.90 (0.06)	0.63 (0.04)	0.28 (0.03)	0.27 (0.04)
Minimum			0.29	0.18	0.0	0
Maximum			1.2	0.97	0.61	0.7

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