Genetic diversity and adaptation in *Eucalyptus pauciflora*

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Abstract

Restoration of degraded land to combat habitat degradation and deforestation requires understanding on adaptive potential of the species. Local adaptation and the geographic scale over which the local adaptation occurs raise issue on how well the existing genotypes will succeed in the face of increasing pressures from climate change and anthropogenic disturbances leading to new environment. This thesis examines genetic factors affecting the success of restoration plantings of the *Eucalyptus pauciflora* subsp. *pauciflora* on the island of Tasmania. Open-pollinated seed and DNA samples were collected from 281 trees from 37 native Tasmanian populations across the distribution and environmental range of the species and used to provide a quantitative and molecular genetics framework to understand local adaptation and guide future environmental planting decisions.

It specifically aims to: i) determine the mating system parameters of *E. pauciflora*, and to explore whether population variation is related to the degree of forest fragmentation or altitude; ii) assess the spatial pattern of genetic diversity in chloroplast and nuclear molecular markers, to understand historical and contemporary barriers to gene flow; iii) explore climate adaptation of the species, through assessing quantitative genetic variation in seedling morphology and growth in a glasshouse trial; iv) determine the effects of inbreeding, local climate and translocation from mainland Australia on genetic variation in performance in Tasmanian field trials up to age 3 years; and vi) provide the seed collection guideline based on the above observations.

Molecular research showed that Tasmanian *E. pauciflora* has a high outcrossing rate (t_m = 0.90). Outcrossing rates differed among populations, but this variation was not correlated with the degree of forest fragmentation nor with altitude. Nevertheless, fragmentation did affect early reproductive output by reducing the number of germinants per gram of capsule content. Chloroplast haplotypes showed clear geographic structure suggesting three low-altitude glacial refugia and recent colonization of high altitude areas. There was little population differentiation in neutral nuclear markers, but populations within 27 km were more similar than average. Similar significant quantitative genetic structure was also detected in the glasshouse trial,

suggesting an operational limit for the definition of a ‘local’ population. Population genetic variation was found for 24 of the 25 seedling traits studied. In several cases this population differentiation exceeded neutral expectations arguing for the action of disruptive selection and that local adaptation has over-ridden historical and contemporary gene flow. This is supported by significant correlations with population altitude and climate variables, with many seedling traits best related to the maximum temperature of the warmest month at the site of origin.

Integrating mating system parameters into the analysis of the two field trials revealed inbreeding depression for growth at the family level, but at the population level outcrossing rate did not affect performance. However, population differentiation was evident for early-age growth, survival, and susceptibility to drought and herbivory. Population differences in early performance appeared to reflect a trade-off between fast growth and herbivore susceptibility, with low altitude populations initially growing faster but rapidly losing their advantage through increased herbivory. Drought and high temperatures at one trial reshaped the fitness profile of the planting, selecting against populations from more moist areas. At both trials the Tasmanian populations outperformed those from the mainland, arguing against the need for seed translocations from mainland Australia.

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Chapter 1. Introduction

1.1 Habitat fragmentation and tree decline

Habitat fragmentation is one of the major threats to biodiversity globally contributing to the decline and extinction of biodiversity worldwide. This partitioning of original vegetation into small isolated fragments, dramatically alters both the biotic and abiotic processes in the landscape (Broadhurst and Young 2007; Hobbs and Yates 2003; Young *et al.* 1996). Coupled with tree decline (Close and Davidson 2004) and rapid climate change, tree populations are being placed under unprecedented pressures. On the one hand, increased deforestation and tree decline have a direct impact on climate change by increasing the overall carbon emission and exacerbating anthropogenic climate change (IPCC 2007), on the other hand pronounced and rapid climate change has a profound impact on the vegetation, including change in current distribution of many tree species (Kremer *et al.* 2014; McKenney *et al.* 2007; Meshinev *et al.* 2000), which may lead to a cycle of tree decline (Bréda *et al.* 2006; Sabaté *et al.* 2002).

Eucalypt forests and woodlands provide vital habitat for a large component of Australia's unique birds and marsupials populations providing nesting sites and hollows for dens and food. Following European settlement, significant components of this habitat have been lost in Australia through clearing for agricultural purposes and tree decline (Yates and Hobbs 1997). Over the last three decades tree decline has reached unprecedented rates (Close and Davidson 2004; Jurskis 2005; Neyland 1996; Rice *et al.* 2004). Although tree decline has been documented in many states of Australia, its severity has been particularly notable on the Tablelands of New South Wales (Jones *et al.* 1990) and the Midlands of Tasmania (Close and Davidson 2004). The Tasmanian midlands have more than 50% tree decline with the aerial extent of severe and extreme tree decline around 30% (Williams *et al.* 2010). Tree decline has several causes including intensive grazing, introduction of improved pasture species, decreased water availability and increasing salinity (Close and Davidson 2004; Kirkpatrick and Bridle 2007; Neyland 1996; Williams *et al.* 2010). This has impacted on the quantity, quality and connectivity of forest habitat through the landscape.

There is increasing interest in tree plantings in the Midlands region of Tasmania for carbon sequestration and biodiversity benefits, including enhancing landscape connectivity for migration corridors (Bailey *et al.* 2013). If the habitat and biodiversity benefits from such tree plantings are to be optimized, it is important that strategies are developed and promoted for the use of local seed source. This might be because the reestablishment of ecosystem forms and function is only possible with the local populations (Lesica and Allendorf 1999). Whether population is local or not is a matter of scale as well as a matter of adaptation (Jones 2003). One of the large concerns in establishment of restoration projects is identifying provenances which are best adapted to these modified or degraded environments (Broadhurst *et al.* 2008; Byrne *et al.* 2011), and likely to succeed in the face of long-term climate change (Hoffmann and Sgrò 2011; Weeks *et al.* 2011).

1.2 Genetic issues in restoration: adaptation versus genetic pollution

Although restoration has been widely used to counter habitat fragmentation and tree decline, the extent to which restoration decisions are affected by the choice of genetic materials used in the restoration is unclear (McKay *et al.* 2005). The use of local genotypes is often viewed as a safe option, as they are considered to be the best adapted to the long term environment of the site and there is less issue with the offsite effects of gene flow and potential mal-adaptation increasing establishment success (Broadhurst *et al.* 2008; Hufford and Mazer 2003; Montalvo *et al.* 1997; O'Brien *et al.* 2007). Several studies have found that local populations can perform better (Goto *et al.* 2011; Linhart and Grant 1996). However, there are often uncertainties on the extent of local adaptation, on what is the geographic scale over which local adaptation occurs, and also whether the existing local genotypes are the best in the face of increasing pressures from climate change and other anthropogenic disturbances leading to a new environment (Byrne *et al.* 2011; Crowe and Parker 2008; Hoffmann and Sgrò 2011; Weeks *et al.* 2011).

There is a risk when using local genotypes in restoration of encouraging populations which do not contain sufficient genetic variation to ensure an ongoing evolutionary potential (Sgrò *et al.* 2011). As local populations in landscape that need restoration are

often severely fragmented and disturbed, there is a risk that local seed may be subject to the effects of small population processes. Such processes include increased inbreeding leading to inbreeding depression, which may eventually override local adaptation as well as genetic drift and loss of genetic variation which can curtail evolutionary potential (Kramer and Havens 2009; Mimura *et al.* 2009; O'Brien and Krauss 2010; Vander Mijnsbrugge *et al.* 2010). Fragmented populations may also be subjected to greater hybridization risk (Field *et al.* 2008) and such invasions may potentially affect evolutionary processes by changing the way genes move around the landscape and thus lead to poor genetic health of local populations (Bischoff *et al.* 2010; Hoffmann and Sgrò 2011; Mortlock 2000). These concerns have raised the idea that alternative seed sourcing strategies such as genetic translocations maybe of potential benefit (Byrne *et al.* 2011; Weeks *et al.* 2011).

Identifying the seed sourcing strategies that can best cope with an ongoing altered environment is a challenging issue in ecological restoration, especially when aiming to restore the past system but at the same time build a resilient system for future changes (Crowe and Parker 2008; Sgrò *et al.* 2011). When considering the use of non-local genotypes in restoration, care must be taken, as choosing the wrong non local genotypes might reduce the success of the restoration project if they are poorly adapted to the new environmental conditions. Furthermore, there is a risk of genetic contamination of local native populations through gene flow from non-local genotypes (Hufford and Mazer 2003; Potts *et al.* 2003). This may result in outbreeding depression or in the worst cases complete genetic swamping (Keller *et al.* 2000; Montalvo *et al.* 1997). Non-local genotypes may even disrupt the local patterns of gene interaction among species through flow-on effects at the community level, thus affecting the ecosystems ability to adapt to future environmental change (Jones 2013). Nevertheless, under some circumstances as discussed above non-local genotypes may have considerable merit over local genotype and their success in a given landscape will depend on the strength of genotype by environmental interactions and the gene flow dynamics of the species (Sgrò *et al.* 2011).

1.3 Evolutionary potential: genetic variation within population, genetic structure, gene flow and adaptability

The capacity of a species to undergo evolutionary adaptation and respond to environmental/climate change depends on the presence of genetic variation within the species (Hoffmann and Sgrò 2011). Therefore, to understand the adaptability of a species or a population, knowledge of the genetic diversity, genetic structure and mating system of the species' is required. The interaction between selection and gene flow determines the adaptive potential of species to their local environment (Davis and Shaw 2001). Gene flow can either promote or restrict local adaptation. The mating system of a species describes its mode of gene transmission across generations which affect the genetic diversity of the offsprings (Fuchs and Hamrick 2011). Hence mating system may affect how populations respond to the environmental change (Levin 2012). Many plants, particularly forest tree species have a mixed mating system (Goodwillie *et al.* 2005; White *et al.* 2007). While most forest tree species are predominantly outcrossing, some degree of self fertilization is usually present and variations in the level of outcrossing can have a major impact on plant fitness due to exposure of deleterious genes leading to inbreeding depression in the product of inbreeding (Eckert *et al.* 2010; Lowe *et al.* 2005). The mating system can be affected by both genetic and ecological factors. These factors include floral structure, self-incompatibility mechanisms and the ecological circumstances of individual flowers, plants, populations and species, including mode of pollination, pollinator type, population size and density and population position in the landscape (Charlesworth 2006; Coates *et al.* 2007).

Anthropogenic fragmentation of populations is one of the important landscape factors known to affect the gene flow of forest trees (Lowe *et al.* 2005; Young *et al.* 1996). Understanding these impacts is important for the management of forest remnants as well as the development of restoration plantings based on local seed sources (Broadhurst *et al.* 2008; Weeks *et al.* 2011). Habitat loss, the reduced stand density and potential impact on changing pollinator's behavior in fragmented population can disrupt gene flow dynamics (Sork and Smouse 2006). This disruption can lead to genetic erosion and increased inter-population divergence, loss of genetic diversity in the

offspring (genetic drift) and increased inbreeding (Bacles and Jump 2011; Lowe *et al.* 2005; Young *et al.* 1996).

There has been considerable research on natural selection and local adaptation in natural plant populations (Alberto *et al.* 2013). However, since many species are used in restoration there is often little information on the species used for restoration and even when some information is available, this usually involves a small set of populations. From a genetic perspective, guiding restoration decisions requires an in-depth understanding of several key issues. The extent and scale to which seed sources are locally adapted is a key issue for restoration purposes, others include the amount of genetic diversity present in the species and how much genetic diversity is required to establish and maintain restoration plantings in the long run (McKay *et al.* 2005). Understanding the patterns of genetic diversity across the species is important as it underscores the response of species to the evolutionary processes operating under current and past environments and helps predict future responses (Neale and Kremer 2011; White *et al.* 2007; Finkeldey *et al.* 2010). The processes of mutation, gene flow, recombination, genetic drift and natural selection shape the patterns of genetic variation within species. A clear understanding of these patterns will help us in developing strategies for the long-term maintenance of plant genetic resources (Rao and Hodgkin 2002) and restoration (McKay *et al.* 2005; Vander Mijnsbrugge *et al.* 2010). This understanding requires knowledge of the patterns of neutral genetic diversity as well as adaptive genetic variation of the species. The pattern of neutral genetic variation reflects the population dynamics and evolutionary processes such as genetic drift, mutation and migration that populations have experienced in the past. Adaptive variation additionally reflects how natural selection has changed local gene pools to adapt to new environments. Genetic information, both molecular and quantitative, can be used to identify the extant and scale of adaptive divergence across species' geographic ranges to inform the choice of source populations for restoration. Many molecular markers such as microsatellites are considered neutral or nearly neutral to selection, so are useful in detecting the patterns of neutral genetic variation (Holderegger *et al.* 2006). Insights into the patterns of adaptive genetic diversity and adaptive potential of a species can be

obtained from quantitative genetic studies of functional traits using common garden experiments (Kremer *et al.* 2014).

1.4 Study system

1.4.1 Choice of species for the study: Eucalyptus pauciflora

Much of the woodlands in the Midlands and Derwent Valley region of Tasmania once occupied by *E. pauciflora* subsp. *pauciflora* are now denuded of trees (Close *et al.* 2010; Kirkpatrick and Bridle 2007). These areas have been subject to extensive tree decline in the last two decades, which is believed to be due to a combination of intensive grazing, introduction of improved pasture species and climate change (Close *et al.* 2010; Kirkpatrick and Bridle 2007; Neyland 1996). This dry, mid-altitude region is an important link between low-altitude and subalpine forest habitats in Tasmania, and there is growing interest in tree plantings in this area to achieve multiple environmental outcomes (Close and Davidson 2002). The University of Tasmania in a collaboration with Greening Australia, has established a series of long-term experiments to study and better optimize the carbon sequestration and biodiversity benefits from restoration plantings in these degraded, agriculturally marginal areas using Tasmanian native species (Bailey *et al.* 2013). As *E. pauciflora* previously occupied the given areas and has been successful in earlier restoration plantings (Close and Davidson 2002; Close *et al.* 2010), these experiments have focused on *E. pauciflora*.

1.4.2 Biology of Eucalyptus pauciflora (Myrtaceae)

1.4.2.1 Phylogeny/Taxonomy

Eucalyptus pauciflora belongs to the genus *Eucalyptus* of Myrtaceae family. *Eucalyptus* is a dominant genus of many Australian woodland and forest ecosystems and comprises more than 700 species (Brooker 2000), most of which are endemic to Australia. The genus *Eucalyptus* is divided into 13 subgenera with the largest two subgenera being *Symphyomyrtus* (500 species approximately) and *Eucalyptus* (100 species approximately) (Brooker 2000). *Eucalyptus pauciflora*, commonly known as the snow gum on mainland Australia and the cabbage/weeping gum in Tasmania, belongs to the section *Cineraceae* of the subgenera *Eucalyptus*. *Eucalyptus pauciflora's* name was coined by Sieber and published by Sprengel (1827) (cited in: Green 1969a). The species

has been documented as comprised of five subspecies (Wiltshire and Potts 2007; Boland *et al.* 2002) while others have reported six subspecies namely subsp. *pauciflora*, subsp. *hedraia*, subsp. *niphophila*, subsp. *parvifructa*, subsp. *acerina* and subsp. *debeuzevillei* (Nicolle 2006b). The subspecies defined for the species varies from author to author. A possible explanation on why it is difficult to define subspecies may be the presence of more or less continuous genetic and phenotypic variation of the species with the altitude (Pryor 1956). The subspecies varies markedly in habitat and morphology (Nicolle 2006b). One of the earliest studies on variation in *E. pauciflora* was carried out by Pryor (1956). Considering subsp. *pauciflora*, subsp. *niphophila*, and subsp. *debeuzevillei*, Pryor (1956), observed a close linear correlation of a number of characters with altitude, for instance decrease in tree height, leaf length, and bark thickness was observed with increasing altitude. However, there is close resemblance of some of the subspecies in many attributes, for example subsp. *pauciflora* and subsp. *parvifructa* closely resemble each other in leaf, bud and fruit morphology but mainly differ in the waxy nature of their buds and fruits (Euclid 2006).

1.4.2.2 Natural distribution and ecology

Eucalyptus pauciflora is one of the most widely distributed eucalypts species in south eastern Australia (Williams 1991), occurring on the mainland as well as the island of Tasmania. Among the six subspecies of *Eucalyptus pauciflora*, subsp. *pauciflora* has the widest geographical distribution, ranging from 28° to 42.5° south. It also grows across a wide climatic range with the mean annual rainfall of its native distribution ranging from approximately 600 to 1900 mm and mean annual temperature ranging from 4.1 to 15.4°C with mean annual temperature of 10.08°C (Boland *et al.* 2002). It occurs on wide range of substrate from both sedimentary and igneous origin.

While *E. pauciflora* usually occurs on well drained soils in cold, dry sub-alpine habitats, it has the ability to withstand very cold temperature, dry winds and periodic drought and combinations of environmental extremes (Boland *et al.* 2002; Williams and Ladiges 1985; Williams and Potts 1996). *Eucalyptus pauciflora* has one of the widest altitudinal ranges of any *Eucalyptus* species (Williams and Ladiges 1985). On the mainland *E. pauciflora* occurs from almost sea level to up to 2000 m altitude where it forms the tree

line on many mountains. The present low altitude populations of the *E. pauciflora* are believed to be relicts from the most recent glacial period, with the species having migrated upslope following post-glacial climate warming (Dodson 1977; Williams 1991; Williams and Ladiges 1985). However, an expansion from high altitude refugia has been suggested as well (Hope and Kirkpatrick 1989).

Widely spread in the cooler parts of Victoria, the species extends northwards across into New South Wales to the southern border of Queensland. It is also found on coastal sites specially in South Gippsland, Mornington Peninsula and to the far south west of Victoria, extending just into the border of South Australia (Nicolle 2006b). *E. pauciflora* subsp. *pauciflora* (hereafter abbreviated to *E. pauciflora*) is the only subspecies reported in Tasmania (Williams and Potts 1996; Boland *et al.* 2002; Williams and Ladiges 1985). In Tasmania the species occurs naturally from near sea-level (10 m) to 1080 m in altitude where it is replaced by the endemic *E. coccifera* as the tree-line eucalypt. Nevertheless, it still occurs over a wide altitudinal range on the island and is a dominant species of many of the forests and woodlands in cold dry areas in the central and eastern part of the island (Williams and Potts 1996). In Tasmania, the species exhibits a preference for Jurassic dolerite substrates and can also occur in coastal sand dunes (Williams and Potts 1996). *Eucalyptus pauciflora* often forms mixed stand with other *Eucalyptus* species. In the lowlands of Tasmania for example it may coexist with *E. ovata* or *E. rubida*, while in the uplands it is usually found with *E. delegatensis*, *E. amygdalina*, *E. dalrympleana* and/or *E. rodwayi* (Williams and Potts 1996). However, in the Snowy Mountains on the Australian mainland, *E. pauciflora* forms pure stands from 1650 m to 1950 m (Pryor 1956).

Eucalyptus pauciflora ranges in habit from stunted mallee at high altitudes to a tall tree at lower altitudes, ranging in height from 6 to 30 m (Nicolle 2006b). *Eucalyptus pauciflora* is heteroblastic to some degree with many traits differing between juvenile and adult leaves (Euclid 2006). Heteroblasty is common in *Eucalyptus*, and the timing of the developmental transition may vary within species and be of adaptive significance (Jordan *et al.* 1999; Lawrence *et al.* 2003; McArthur and Potts 2006; Wiltshire *et al.*

1998). *Eucalyptus pauciflora* is easily identified from other eucalypt species, especially in Tasmania, due to the distinctive parallel veins evident in its adult leaves.

Fig. 1.1. Left panel shows a typical mature tree of *E. pauciflora* in the woodlands near Derwent Bridge, Tasmania. On the right is a *E. pauciflora* flower (Flower photo courtesy Dr. Tanya Bailey).

1.4.2.3 Reproductive biology

As with all eucalypts, *E. pauciflora* has a haploid chromosome number of 11 (Grattapaglia *et al.* 2012). The chloroplast genome of eucalypts is maternally inherited (Byrne *et al.* 1993; McKinnon *et al.* 2001a), and while not specifically studied, this is likely also the case in *Eucalyptus pauciflora* as it is in most angiosperms (Petit *et al.* 2005; Reboud and Zeyl 1994). *Eucalyptus pauciflora* produces small white flowers in a simple axillary inflorescence with clusters of between 7-15 flowers per inflorescence (House 1997). Most eucalypt flowers are bisexual thus allowing the opportunity for selfing via geitonomy. At low altitudes flowering occurs in early spring but is delayed at higher altitudes, occurring during mid summer around the end of December (Duncan 1989; Pryor 1976). Similar to many other eucalypt species, *E. pauciflora* is predominantly outcrossing but a relatively high level of selfing has been reported (Phillips and Brown 1977). The pollination biology of the species has not been studied, but as with most *Eucalyptus* with small white flowers it is likely to be pollinated by a diverse range of insects with less frequent visitations by birds and mammals (House 1997).

Hybridization plays an important evolutionary role in plants in speciation (see Rieseberg and Ellstrand 1993) as well as dispersal (Potts and Reid 1988). Natural hybridization between closely related eucalypts is common, but the extent of hybridization is limited to within subgenera (Griffin *et al.* 1988; Potts and Wiltshire 1997). *Eucalyptus pauciflora* hybridizes with many other closely related species that can be observed near it. For example, hybrids between *E. pauciflora* and *E. dives* are quite common when the species co-occur (Pryor 1951; Pryor 1976). Natural hybridization of *E. pauciflora* and the co-occurring peppermint species *E. fastigata*, *E. robertsoni*, *E. radiata*, and *E. rossii* has also been observed in numerous field examinations in the Southern Tablelands (Pryor 1951; Pryor 1953; Whiffin 1981). A study of seedling character segregation has also indicated hybridization between *E. pauciflora* and *E. obliqua* and between E. *pauciflora* and *E. radiata* (Williams and Ladiges 1985). Natural hybridization of *E. pauciflora* with *E. delegatensis*, *E. amygdalina*, *E. nitida*, *E. pulchella* and *E. coccifera* has been observed in Tasmania (Duncan 1989).

1.4.2.4 Genetic variation in *Eucalyptus pauciflora*

In the early and mid 90s, there have been several studies on genetic variation in physiological and morphological traits (Ferrar *et al.* 1989; Harwood 1980; Harwood 1981; Pryor 1951; Pryor 1953; Pryor 1956; Pryor 1961; Pryor 1976; Slatyer 1977a; Slatyer 1977b; Slatyer 1977c; Slatyer 1978; Slatyer and Ferrar 1977a; Slatyer and Ferrar 1977b; Slatyer and Morrow 1977; Williams and Ladiges 1985) of the species. One of the earliest studies on population variation in morphology within *E. pauciflora* was done by Pryor (1956). Using mainland *E. pauciflora* populations, Pryor (1956) observed a altitudinal adaptation of tree form, growth and morphology. Similarly, genetic based adaptation of increasing frost resistance, (Green 1969b; Harwood 1980; Harwood 1981) and photosynthetic physiology (Slatyer 1977a; Slatyer and Ferrar 1977a) to increasing altitude has also been reported. Most of these studies were based on the mainland *E. pauciflora* populations. In addition, there have been no studies of genetic variation of *Eucalyptus pauciflora* using molecular methods published to date.

1.5 Thesis structure, objectives and hypothesis

The thesis investigates the mating system and population genetic variation in *E. pauciflora* with a focus on Tasmanian populations, relevant to restoration work. Chapter 1 deals with the general introduction and the background to the thesis. It also comprises thesis structure and overall aim of the study. Chapters 2 to 5 are experimental chapters presented in the format of publishable papers. Hence repetition of concepts and ideas particularly in introduction sections was unavoidable. Chapter 6 synthesizes the experimental work and summarizes the key finding of this study and implications for restoration.

The motivation of the thesis is to link research with the theory of adaptation within the framework of forest restoration. This research project aims to determine the importance of genetic factors in determining the success of restoration planting of *E. pauciflora* as well as provide a morphological/ molecular genetics and environmental framework to interpret the adaptive responses observed in the field trial and guide future environmental planting decisions. The thesis is aimed to provide information to underpin seed collection guidelines and restoration decisions. Specific objectives of the study were:

- to determine the mating system parameters of *E. pauciflora* and variation pattern among the populations, and to explore whether population variation is related to the degree of forest fragmentation or altitude of origin;
- to explore the spatial pattern of genetic diversity in chloroplast and nuclear molecular markers and to understand historical and contemporary barriers to gene flow;
- to assess the adaptive potential of the species through assessing quantitative genetic variation in seedling morphology and growth across the native range of the species in Tasmania;
- to determine the effect of inbreeding, local climate and translocation from mainland Australia on genetic variation in performance in Tasmanian field trials up to age 3 years; and
- to inform seed collection guidelines based on the above observations.

Specific hypotheses tested include:

- Populations vary in their mating system estimates and the variation in mating system parameters is affected by fragmentation and altitude.
- As *E. pauciflora* is a widespread species, historically had large population sizes, and occupies diverse habitats, there will be little neutral molecular genetic differentiation amongst populations in Tasmania but significant genetic variation in adaptive traits.
- Populations and families with higher outcrossing rate will be less affected by inbreeding depression and hence will perform better in the field trials.
- Locally collected seeds might not always be better adapted (as measured by better growth and survival) than the seedlings raised from non-local seeds collected from intact native forest because of a combination of inbreeding and changing environments.

Chapter 2 estimates mating system parameters of the species and determines the pattern of mating system variation among the populations. It explores the variation in mating system parameters across the species range to determine if it is spatially structured and if so, whether it will be predicable from either the altitude of origin or the degree of fragmentation. The mating system of the species provides insights into pollen-mediated gene flow. Three estimates dominates the description of the plant mating system namely outcrossing rate (t_m), the level of biparental inbreeding (t_m-t_s) and correlated paternity (r_p, estimated as a probability that two randomly chosen offspring within an open-pollinated family are full-sibs).

Chapter 3 describes the pattern of neutral genetic diversity in molecular markers and spatial distribution of the diversity across the native range of *E. pauciflora* in Tasmania. Maternally inherited chloroplast microsatellites are used to explore chloroplast haplotype diversity and variation among populations. Spatial haplotype diversity and the distribution of the haplotypes are used to infer the genetic signature of past climate change on the species distribution. Biparentally inherited and putatively neutral nuclear markers, were used to infer a more recent perspective on gene flow patterns and the level of population-level inbreeding due to genetic drift. The comparison of both

chloroplast and the nuclear microsatellites are made to have insights into the relative importance of seed- versus pollen-mediated gene flow.

Chapter 4 explores the extent to which selection may have shaped the patterns of genetic variation in *E. pauciflora* through assessing seedling traits using a glasshouse trial. Evidence of selection having shaped variation in seedling traits is obtained from comparing the molecular and quantitative genetic estimates of population-level inbreeding, as well as looking for associations between population variation and climate variables at the univariate and multivariate level. The chapter addresses the extent to which these traits are genetically correlated and whether these correlations are likely to constrain or enhance the selection process.

Chapter 5 determines whether there is genetic variation in early-age growth and survival in field trials at the population and family level. Growth, survival and other fitness related traits were recorded on a regular basis from pedigreed field trials over their first three-years after establishment. The chapter evaluates the issue of seed source choice-local versus non local for restoration purposes.

Chapter 2. The effect of forest fragmentation and altitude on the mating system of *Eucalyptus pauciflora*

Abstract

Habitat fragmentation is a key factor causing variation in important mating system parameters in plants, but its effect is variable. We studied mating system variation among 276 native trees from 37 populations of *Eucalyptus pauciflora* from Tasmania. We assayed 10 microsatellite loci from 1,359 open pollinated progeny from these trees. Across Tasmania, the species' mating system was characterized by a high outcrossing rate (tm = 0.90) but moderate bi-parental inbreeding (tm-ts = 0.16) and moderate correlated paternity (rp = 0.20) in comparison to other eucalypt species. Despite significant differences in outcrossing rate and correlated paternity among populations, this variation was not correlated with fragmentation. Nevertheless, fragmentation was inversely correlated with the number of germinants per gram of seed capsule content. Outcrossing rate had been reported previously to decrease with increasing altitude in mainland populations of *E. pauciflora*, but this was not the case in Tasmania. However, a small but significant decrease in correlated paternity occurred with increasing altitude and a decrease in bi-parental inbreeding with increasing altitude was evident in fragmented populations only. It is argued that strong but incomplete self-incompatibility mechanisms may buffer the mating system from changes in population density and pollinators. While seed yields from highly fragmented populations were reduced, in most cases the seed obtained is unlikely to be more inbred than that from non-fragmented populations and, thus, is likely to be as suitable for use in local forest restoration.

2.1 Introduction

The mating system of plants plays an important role in determining the distribution of genetic variation within and among populations (Charlesworth 2006; Hamrick and Godt 1989). The mating system determines the mode of gene transmission across generations which, in turn, affect the genetic composition of the progeny (Fuchs and Hamrick 2011). Many plants, particularly forest tree genera, have mixed mating systems (Goodwillie *et*

al. 2005; White *et al.* 2007). The rate of selfing within a species can have major impacts on population fitness through the exposure of deleterious alleles that can lead to inbreeding depression (Eckert *et al.* 2010; Lowe *et al.* 2005). Mating system can be affected by genetic factors, including floral structure and self-incompatibility mechanisms, as well as the ecological circumstances of individual flowers, plants, populations and species, the mode of pollination, pollinator type, population size, population density and the position of a population in the landscape (Charlesworth 2006; Coates *et al.* 2007).

Fragmentation of populations is one of the key landscape factors known to affect the mating system of forest trees (Lowe *et al.* 2005; Young *et al.* 1996). Understanding the impact of fragmentation of anthropogenic origin is important for the management of forest remnants as well as the development of seed sourcing policy for ecological restoration based on local seed sources (Broadhurst *et al.* 2008; Weeks *et al.* 2011). In recently fragmented populations, habitat loss and reduced stand density, as well as the potential impact of these on pollinator behaviour, can disrupt gene flow dynamics (Sork and Smouse 2006). These can lead, in turn, to genetic erosion (loss of genetic diversity), increased inter-population divergence (genetic drift) and increased inbreeding (Bacles and Jump 2011; Lowe *et al.* 2005; Young *et al.* 1996). While many studies have demonstrated such deleterious effects of fragmentation (Aguilar *et al.* 2008; Hamrick 2004; Mimura *et al.* 2009), several studies have shown that genetic diversity can be maintained through extensive outcrossing and long distance pollen movement among even quite distant fragments (Byrne *et al.* 2008; Schuster and Mitton 2000). The impact of fragmentation is determined by the amount of gene flow, the diversity of the pollen pool and the mating system (Kramer *et al.* 2008; Sork and Smouse 2006). The presence and strength of self-incompatibility mechanisms in a species affect the impact of fragmentation. Some self-compatible species may be resistant to inbreeding depression, having already experienced genetic bottlenecks that have eliminated many deleterious alleles (Husband and Schemske 1996; Kramer *et al.* 2008). While it is commonly assumed that fragmented populations should be avoided as seed sources for restoration due to deleterious effects on the mating system (Broadhurst *et al.* 2008), there is variation in the effect of fragmentation on mating system and more studies are required

to better understand the effects of fragmentation and consequences for seed sourcing decisions.

Many of Australia's tree restoration programs are focused on species of the genus *Eucalyptus* (Bradbury and Krauss 2013; Krauss *et al.* 2007; O'Brien *et al.* 2007). *Eucalyptus* species are normally pollinated by generalist animal vectors, particularly insects and birds (House 1997) and, while they have a mixed mating system, they are predominantly outcrossing (Byrne 2008b; Horsley and Johnson 2007; Potts and Wiltshire 1997). The hermaphrodite flowers are protandrous in their development which reduces the probability of self-pollination (House 1997), but the asynchrony of flower development throughout the canopy facilitates geitenogamous pollination (Byrne 2008b). Allozyme studies of mating systems in eucalypts have demonstrated variable, but generally high, outcrossing rates ranging from 0.51 to 0.96 and averaging 0.74 across 23 species (Byrne 2008b). The high outcrossing rates may be explained by a combination of pre- and post-zygotic self-incompatibility mechanisms (Ellis and Sedgley 1992; Horsley and Johnson 2007; Pound *et al.* 2002). These endogenous self-incompatibility mechanisms are obviously incomplete in most eucalypt species, but are reinforced by post-dispersal selection against inbred progeny (McDonald *et al.* 2003; Potts and Wiltshire 1997). Inbreeding depression for growth and survival following selfing has been reported for several species (Costa e Silva *et al.* 2010). Forest fragmentation has been reported to adversely affect the mating system of several eucalypt species, through increased selfing rates (Butcher *et al.* 2005; Hardner *et al.* 1996; Millar *et al.* 2000; Mimura *et al.* 2009), but this is not always the case (Breed *et al.* 2012b; Broadhurst 2013). Management guidelines for forest restoration usually favour the use of locally collected seed (Mortlock 2000). However, with increasing interest in forest restoration for biodiversity and carbon sequestration (Broadhurst *et al.* 2008), a greater understanding of the effects of forest fragmentation on the mating systems of eucalypts is required, because increased inbreeding may reduce the success of restoration plantings established from seed collected from local forest remnants (Borralho and Potts 1996).

The present study focuses on *Eucalyptus pauciflora*, an iconic forest and woodland tree of south-eastern Australia (Boland *et al.* 2002). It is one of the most widely distributed eucalypt species in Australia, occurring at the tree-line on most of the Australian Alps and extending to near sea level in southern (Victoria and on the island of Tasmania (Boland *et al.* 2002; Williams 1991; Williams and Potts 1996). This natural range includes valley bottoms in the midlands of Tasmania, where these once extensive woodlands have been reduced to isolated fragments as a result of agricultural land clearing, intensive grazing and climate related tree decline (Close *et al.* 2010; Kirkpatrick and Bridle 2007). Owing to the species' ability to cope with harsh environmental conditions and its success in restoration species trials (Close and Davidson 2002; Close *et al.* 2010), *E. pauciflora* has been targeted as a key tree species for use in ecological restoration and carbon plantings in Tasmania (Bailey *et al.* 2013).

Eucalyptus pauciflora is animal pollinated and, while having a mixed mating system, is predominantly outcrossing. The only published mating system study compared allozyme profiles in seeds and seedlings from three mainland populations that spanned an altitudinal gradient (Phillips and Brown 1977). The study observed reduced outcrossing at higher altitudes and provided evidence of early age selection against the products of self-fertilisation. However, the ubiquity of these trends is unknown and there is no data on the impact of habitat fragmentation on the outcrossing rate of the species. In the present study, we assess the outcrossing rate of *E. pauciflora* using microsatellite markers. We focus on the Tasmanian populations of *E. pauciflora* which are being used as seed sources for ecological restoration and carbon plantings. Specifically, we aim to: (i) provide species-wide estimates of mating system parameters for Tasmania, (ii) identify whether these parameters vary between populations and, if so, (iii) determine whether the variation is spatially structured and predictable from either the altitude of origin or the degree of fragmentation. Such basic information will form the foundation of further studies of genetic diversity in the species and allow assessment of the value of fragmented populations as seed sources for ecological restoration (Broadhurst *et al.* 2008; Sgrò *et al.* 2011; Weeks *et al.* 2011).

2.2 Materials and methods

2.2.1 Sample collection

Leaf samples for DNA extraction and seed capsules were collected from 5 to 10 trees from each of 37 populations representing the entire geographic and climatic distribution of *E. pauciflora* in Tasmania (Table 2.1). In total, 281 trees were sampled from the wild. To avoid sampling closely related individuals, a minimum distance of 100 m separated the sampled trees. This was more than double the average tree height and would transgress any family group structure in the forest (Jones *et al.* 2007; Skabo *et al.* 1998). Geographic coordinates and altitude were recorded for each tree. Sampled populations were classified into four stand types based on the classification system of Borralho and Potts (1996): i) stand type I, isolated trees; ii) stand type II, few trees in a small isolated patch; iii) stand type III, trees in open stands of continuous distribution; and iv) stand type IV, trees in closed stands of continuous distribution. This classification was applied in the field at both the population level and at the individual tree level to account for local variation in tree density within a population. Leaf samples were dried with silica gel crystals and sealed in aluminium foil bags for long term storage.

2.2.2 Progeny growth and sampling

The 281 families derived from the open-pollinated seed collection were used to establish a progeny trial embedded in restoration plantings of *E. pauciflora* at Dungrove (-42° 16' 29.3052" S, 146° 53' 28.0098" E) (Bailey *et al.* 2013). For each seedlot, a measured weight of capsule content comprising both seed and chaff (Boland *et al.* 1980), was soaked in water overnight, drained, and then stratified at 4°C for 4 weeks (from 25th January 2010). Each seedlot was then sown onto soil in germination trays and allowed to germinate at room temperature in a commercial nursery. After 8 weeks, the number of germinants per gram of capsule content sown was recorded for each seedlot. Germinants were pricked out (9th to 19th March 2010) into individual cells in Hyko seedling trays; each tray contained 40 plants of one family. Family tray positions were then randomised in an indoor growing area of a nursery and transferred outside after 10 weeks. Eight months after sowing, the number of seedlings surviving per tray was recorded. Seedlings were then used to establish a field trial (date of planting: 5th October 2010) in which families were randomised into a resolvable row (20) X column (20)

Table 2.1. Mating system parameters for 37 populations of *Eucalyptus pauciflora.* Population location (latitude, longitude, altitude), stand classification (SC; Borralho and Potts 1996) and number of maternal trees (Nm) and progenies (Np) sampled per population are shown. Multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), bi-parental inbreeding (t_m-t_s), correlated paternity (r_p) and effective number of pollen donors ($1/r_p$) were estimated from offspring and the maternal parent genotypes using the Expectation-Maximization method (MLTR version 2.4; Ritland 2002). Standard error (S.E.) is provided for each estimate.

Code	Population	Latitude	Longitude	Altitude	SC	Nm	Np	t_m	S.E (t_m)	t_s	S.E (t_s)	$t_m - t_s$	S.E ($t_m - t_s$)	r_p	S.E (r_p)	$1/r_p$
1	Waterhouse	-40.9098	147.65993	16	2	6	39	0.98	0.003	0.82	0.051	0.17	0.05	0.17	0.10	5.81
2	Nunamara	-41.3728	147.32153	405	3	8	39	0.87	0.040	0.79	0.049	0.08	0.03	0.11	0.04	9.17
3	Brushy Lagoon	-41.4094	146.74695	282	4	7	38	0.80	0.086	0.82	0.061	-0.01	0.04	0.07	0.02	14.08
4	Tyne River	-41.4709	147.81712	297	3	6	29	0.89	0.049	0.75	0.058	0.14	0.05	0.19	0.05	5.18
5	Longford	-41.6302	147.09729	159	2	10	37	0.91	0.041	0.76	0.033	0.16	0.03	0.26	0.05	3.82
6	Symmons Plains	-41.6594	147.2491	166	2	6	38	0.89	0.085	0.79	0.042	0.10	0.06	0.24	0.07	4.17
7	Rossarden	-41.6888	147.69499	731	4	7	40	0.97	0.005	0.86	0.044	0.11	0.05	0.18	0.14	5.52
8	Avoca	-41.7095	147.83446	237	2	6	39	0.98	0.003	0.86	0.024	0.12	0.03	0.18	0.05	5.52
9	Cressy	-41.7193	147.10455	160	2	8	38	0.97	0.005	0.85	0.021	0.12	0.02	0.19	0.04	5.18
10	Lake Rowallan	-41.7218	146.21847	460	3	8	39	0.94	0.024	0.88	0.023	0.06	0.02	0.07	0.03	13.51
11	Dukes Marshes	-41.7225	148.12791	498	3	8	38	0.90	0.044	0.80	0.037	0.10	0.04	0.09	0.02	11.63
12	Conara	-41.8416	147.46348	206	3	8	39	0.98	0.003	0.85	0.014	0.12	0.02	0.18	0.03	5.59
13	Lake Arthur	-41.9565	146.87693	1004	4	9	36	0.90	0.057	0.85	0.021	0.04	0.04	0.07	0.02	13.89
14	Great Lake	-41.9868	146.69913	1138	3	10	37	0.90	0.030	0.84	0.036	0.07	0.02	0.06	0.01	17.54
15	Ross	-42.0017	147.53229	240	3	9	37	0.89	0.040	0.87	0.018	0.02	0.03	0.08	0.01	12.50
16	Lake Leake	-42.0211	147.81729	597	4	9	39	0.95	0.024	0.88	0.015	0.07	0.02	0.08	0.02	12.05
17	Wihareja	-42.0614	146.81432	895	2	7	39	0.89	0.035	0.88	0.020	0.01	0.03	0.26	0.05	3.89
18	Pine Tier	-42.0937	146.51663	818	4	8	36	0.94	0.025	0.85	0.025	0.09	0.03	0.11	0.02	9.35
19	Tunbridge	-42.1249	147.36459	229	2	9	38	0.94	0.023	0.83	0.024	0.11	0.02	0.20	0.05	5.05
20	Interlaken	-42.1461	147.14116	818	2	8	37	0.97	0.004	0.89	0.010	0.08	0.01	0.10	0.04	10.20
21	The Point	-42.1929	146.42217	674	2	7	38	0.91	0.025	0.87	0.013	0.04	0.02	0.07	0.01	14.93
22	Lake St Clair	-42.2014	146.14225	816	3	9	36	0.97	0.003	0.89	0.007	0.08	0.01	0.07	0.02	13.51
23	Woodbury Hill	-42.2124	147.28282	626	3	5	29	0.88	0.072	0.84	0.040	0.04	0.04	0.08	0.03	11.90
24	Tooms Lake	-42.2205	147.79278	487	4	5	40	0.80	0.087	0.75	0.108	0.05	0.10	0.31	0.13	3.18
25	Dungrove	-42.2664	146.88613	552	2	10	37	0.84	0.072	0.75	0.051	0.09	0.05	0.17	0.04	5.88
26	Butlers Gorge	-42.2792	146.33043	682	4	8	39	0.93	0.029	0.84	0.025	0.09	0.02	0.09	0.02	11.63
27	Oatlands	-42.3013	147.38423	402	1	8	39	0.81	0.056	0.80	0.032	0.01	0.04	0.15	0.02	6.67
28	Tin Dish Rivulet	-42.3079	147.43698	412	3	6	39	0.76	0.078	0.77	0.065	-0.01	0.03	0.08	0.02	12.66
29	Osterley	-42.3543	146.74082	347	2	4	21	0.84	0.100	0.85	0.049	-0.01	0.06	0.06	0.01	16.95
30	Bothwell Lake	-42.3798	146.99545	370	2	10	38	0.91	0.040	0.88	0.022	0.04	0.02	0.06	0.02	16.39
31	Bignells Bothwell	-42.4014	147.09624	481	2	7	34	0.96	0.022	0.87	0.027	0.08	0.03	0.27	0.09	3.68
32	Ellesmere	-42.4014	147.29766	422	1	5	37	0.77	0.082	0.81	0.058	-0.04	0.03	0.26	0.07	3.83
33	Stonor	-42.4277	147.43164	444	2	8	37	0.84	0.045	0.84	0.033	0.00	0.02	0.17	0.07	6.02
34	Uralla	-42.5462	146.85911	193	1	5	29	0.97	0.006	0.85	0.044	0.12	0.05	0.14	0.06	7.14
35	Curringa	-42.5698	146.77209	100	2	8	38	0.81	0.061	0.77	0.056	0.04	0.03	0.08	0.02	12.99
36	Gatehouse Marsh	-42.5949	147.78101	41	2	6	36	0.75	0.072	0.72	0.063	0.03	0.05	0.25	0.05	4.02
37	South Arm	-43.0341	147.42227	16	2	8	40	0.96	0.016	0.76	0.063	0.21	0.05	0.38	0.11	2.66
	Overall species					276	1359	0.90	0.013	0.74	0.015	0.155	0.013	0.20	0.022	4.95

design with eight replicates using CycDesigN 4.0 (Whitaker *et al.* 2002). Families were represented as single-tree plots within each replicate. After 4 months of growth in the field, seedling survival was assessed and 1-2 leaves were collected from each survivor for molecular studies. Sampling was undertaken in such a way that each of the 37 populations was represented by 40 seedlings from 5 - 8 families, giving a total of 1480 seedlings. When sampling, priority was given to the replicates within the field trial that had the lowest mortality; replicates were sampled until the required number of seedlings per population was obtained. Leaf samples were freeze-dried and sealed in aluminium foil bags until DNA extraction was undertaken.

2.2.3 DNA extraction and microsatellite analysis

Genomic DNA was extracted from maternal trees and progenies using a modified CTAB method (Doyle and Doyle 1990; McKinnon *et al.* 2004b). Fifteen nuclear microsatellites were screened: CRC6, CRC11 (Steane *et al.* 2001), EL13 (Ottewell *et al.* 2005), ES140, ES157, ES211, ES255 (Glaubitz *et al.* 2001), EMBRA08, EMBRA011, EMBRA042, EMBRA187, EMBRA196, EMBRA210, EMBRA232 (Brondani *et al.* 1998; Brondani *et al.* 2006) and EPIL_MYB2 (Shepherd *et al.* 2010). Two loci EMBRA042 and EPIL_MYB2 did not amplify and three loci (EMBRA08, CRC6 and ES157) yielded many spurious peaks that made scoring difficult, so these five loci were excluded from further analysis. The forward primer for each locus was labelled with NED, VIC, 6-FAM, or PET fluorescent dyes (Perkin Elmer Applied Biosystems, Foster City, CA, USA). PCRs were performed in 5 µl reactions containing approximately 1 µl of 20 ng DNA, 2.5 µl of 2X QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$), 0.5 µl of 5X Q-Solution, and 0.1 µl of Primer mix containing 10µM of each forward and reverse primer.

The PCR profile consisted of 15 min denaturation at 95 °C followed by 30 cycles of 94 °C for 30 sec, 57 °C for 90 sec and 72 °C extension for 60 sec, followed by a final extension for 10 min at 60 °C. PCR products were sent to the Australian Genome Research Facility Ltd for capillary separation on an AB3730 analyser (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Alleles were sized and scored using Genemapper software version 3.7 (Perkin Elmer, Applied Biosystems) and these results were checked visually. MLTR version 3.4 (Ritland 2002) was used to further check for

genotyping errors. Progeny with microsatellite profiles that did not have at least one maternal allele at all loci, were removed from the analysis. Maternal samples from the South Arm population were not available for the study so MLTR was used to predict maternal genotypes using genotype information from their progeny. These simulated genotypes were used in subsequent analyses. Five families and their parents were excluded from further analyses because technical problems (e.g. failed DNA extractions, failed PCRs or poor allele resolution) resulted in excessively small sample sizes. This left 276 families and 1635 individuals (including maternal samples) available for analysis.

2.2.4 Mating system analysis

Maximum likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates from offspring and the seed parent genotypes based on mixed mating analysis (Ritland 2002) were estimated. The level of bi-parental inbreeding was estimated as the difference between the multilocus and the single-locus outcrossing rates (t_m-t_s; Ritland 2002). The correlation of outcrossed paternity within a progeny array was estimated as r_p, which corresponds to the probability that two randomly chosen progenies share the same pollen donor and, thus, are full sibs. An estimate of the effective number of pollen donors was obtained as $1/r_p$ (Sun and Ritland 1998). MLTR version 2.4 (Ritland 2002) was used to estimate all mating system parameters. Population level maximum likelihood parameters for each population were estimated using both Newton Raphson (NR) and Expectation Maximization (EM) methods. The multilocus outcrossing values were greater than 1 for some populations, and r_p estimates had higher standard errors and occasional negative parameters when the NR method was used. The EM method was therefore chosen to estimate both overall and population level mating system parameters. Overall estimates were obtained ignoring population structure. Standard errors for population and family level parameter estimates were calculated using 1000 bootstrap replicates. We considered differences in estimates to be statistically significant if bootstrap-derived standard error estimates did not overlap. Family level estimates of t_m and t_s were obtained using the method of 'moments procedure' within MLTR (Ritland 2002) and were used to test for the effects fragmentation (see below).

2.2.5 Statistical analysis

The differences between populations and the effect of fragmentation on mating system parameters (t_m, t_s and t_m-t_s), number of germinants per gram of capsule content, and nursery and field survival were tested using family-level values and the non-parametric Kruskal-Wallis test. The correlation between these traits was examined using Pearson's correlation coefficients. Matrices of the pair-wise differences between populations were generated for outcrossing rate, number of germinants per gram of capsule content and geographic distance. The geographic distances among populations were calculated from differences in latitude and longitude (Table 2.1), which, for each population, were the average of the tree GPS coordinates. These matrices were used in Mantel tests (Mantel 1967) and spatial autocorrelation analyses using GENALEX 6.501 (Peakall and Smouse 2006). The effect of population altitude on mating system parameters and number of germinants was analysed using linear regressions and population-level data. The effect of forest fragmentation on mating system parameters and number of germinants was tested using the stand type score and the non-parametric Kruskal-Wallis test applied with both population and family-level data. In the case of the mating system parameters, population level estimates were derived from the EM methods, as described above. The non-parametric Kruskal-Wallis tests and regressions were undertaken using standard functions in R (Team 2010). Whether the effect of altitude differed amongst stand types was tested by analysis of covariance fitting a model with altitude (covariate), stand type (fixed) and their interaction using PROC MIXED of SAS (version 9.1; SAS Institute) and the population-level data.

2.3 Results

Eucalyptus pauciflora had an overall multilocus outcrossing rate (t_m) of 90%, with population estimates ranging from 75% (Gatehouse Marsh) to 98% (Waterhouse) (Table 2.1). Single locus outcrossing estimates (t_s) were generally lower than the multilocus outcrossing rates, indicating overall significant bi-parental inbreeding (t_m-t_s = 0.16±0.013). Population estimates of bi-parental inbreeding ranged from -0.04 (Ellesmere) to 0.21 (South Arm), with standard errors indicating that most values were significantly greater than zero (21 populations out of 37). The overall correlation of paternity amongst open-pollinated progeny of the same family was also significantly greater than zero (r_p = 0.20±0.013), ranging from 0.06 to 0.38.

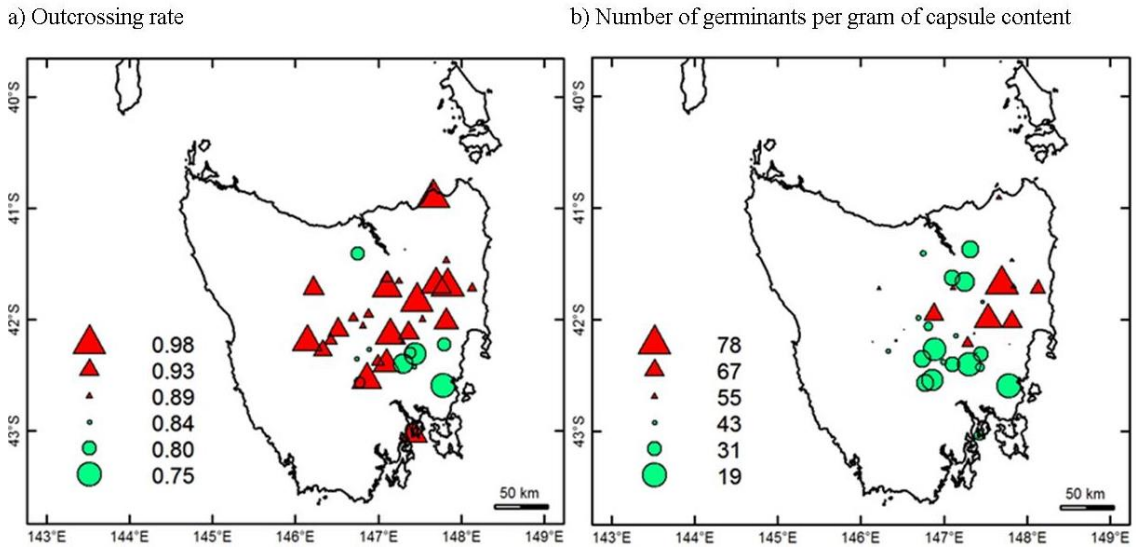

Fig. 2.1. Geographic variation of a) outcrossing rate, and b) number of germinants per gram of capsule content among 37 *Eucalyptus pauciflora* populations. Lower outcrossing rates were observed in the south-east of the distribution with significant variation ($P < 0.01$) among populations. Note that central-eastern populations tended to have a higher reproductive output.

The multilocus outcrossing rates differed significantly ($P < 0.01$) among populations (Table 2.2). Despite a tendency for lower outcrossing rates in populations in the south-east of the distribution (Fig. 2.1a), the Mantel test ($r = 0.05$, $P = 0.243$) and the spatial autocorrelation analysis (result not shown) revealed no statistically significant spatial structure. In addition there was no significant association of the multilocus outcrossing rate with altitude (Table 2.2). There was a trend for bi-parental inbreeding to increase with decreasing altitude ($P < 0.1$; Table 2.2). Bi-parental inbreeding was the only parameter for which a significant interaction between altitude and stand type was detected ($F_{3,29} = 3.2$, $P < 0.05$). This interaction was due to the altitudinal effect only being evident in the fragmented stand types I and II. These fragmented stand types also tended to be more common at lower altitudes ($F_{3,33} = 2.7$, $P = 0.061$). While family level-estimates of correlated paternity and the effective number of pollen donors were not available to test for population differences, the standard errors of their estimates clearly indicated significant differences among populations (Table 2.2). The Mantel test ($r = 0.14$, $P = 0.051$) and the spatial autocorrelation analysis showed no statistically significant spatial structure in correlated paternity. However, regression analysis revealed that correlated paternity decreased, and thus the effective number of pollen donors increased, with increasing altitude (Table 2.2). Altitude explained 15.4% and

17.4% of the variation between populations in correlated paternity and effective number of pollen donors, respectively. No significant effect of stand type on outcrossing rate or on any other mating system parameter was detected, regardless of whether analyses were done using population-level estimates (Table 2.2) or family-level estimates (t_m: Kruskal-Wallis $\chi^2_3 = 2.2$, $P = 0.54$; t_m-t_s: Kruskal-Wallis $\chi^2_3 = 5.6$, $P = 0.132$).

Table 2.2. Mating system parameters and number of germinants per gram of capsule content estimated across 37 populations of *Eucalyptus pauciflora*. Overall and population level estimates were obtained using the Expectation-Maximization method. Overall estimates were obtained ignoring population structure. Population level parameter estimates were used for the regression analysis to test the effect of altitude on populations. Kruskal-Wallis χ^2 test were performed to test the effect of population stand types and altitude on the mating system and seed yield. Stand type classification was applied to each population.

Parameter	Overall[a]	Population		Stand_types		Altitude			
		χ^2_{36}	*P*	χ^2_3	*P*	R^2 (%)	Relationship	$F_{1,35}$	*P*
Multilocus outcrossing rate, t_m	0.90 (0.013)	62.3	0.004	1.0	0.810	1.2	ns	0.4	0.523
Single locus outcrossing rate, t_s	0.74 (0.015)	47.2	0.100	0.4	0.942	4.2	positive	9.8	0.003
Bi-parental inbreeding, t_m-t_s	0.16 (0.013)	47.5	0.095	1.4	0.703	7.9	ns	3.0	0.091
Correlated paternity, r_p	0.20 (0.022)		b	5.8	0.122	15.4	negative	6.4	0.016
Effective number of pollen donors, 1/ r_p	4.95		b	5.4	0.147	17.4	positive	7.4	0.010
Number of germinants per gram of capsule content	43.5 (15.2)	83.1	0.001	3.5	0.019	9	ns	3.7	0.07

[a]For overall t_m, t_s, t_m-t_s and r_p, standard errors are given in parentheses. For overall number of germinants per gram of capsule content, standard deviation is given in parentheses.
[b]Family level estimate could not be obtained to undertake Kruskal-Wallis test, however, population differences were significantly different based on the standard error (Table 2.1).

Populations differed significantly ($P < 0.001$) in the number of germinants per gram of capsule content (Table 2.2), with a four-fold difference across populations. Populations in the central-east of the range tended to have higher values (Fig. 2.1b), but this spatial structuring was not statistically significant (Mantel r = 0.14, $P = 0.062$). The number of germinants per gram of capsule content tended to increase with altitude, but again this

was not significant (P = 0.070). There was, however, a highly significant effect of stand type on the number of germinants per gram of capsule content obtained, using population-level estimates (Table 2.2) or family-level estimates (Kruskal-Wallis χ^2_3= 21.6, P < 0.001). The average number of germinants per gram of capsule content decreased with increasing stand fragmentation, with a significant reduction in number of germinants obtained from Stand Types I and II compared with Stand Type IV (Fig. 2.2). Despite the absence of a significant effect of stand type on the multilocus outcrossing rate (t_m), there was a weak positive correlation of the number of germinants with t_m (Pearson r= 0.42, P = 0.009).

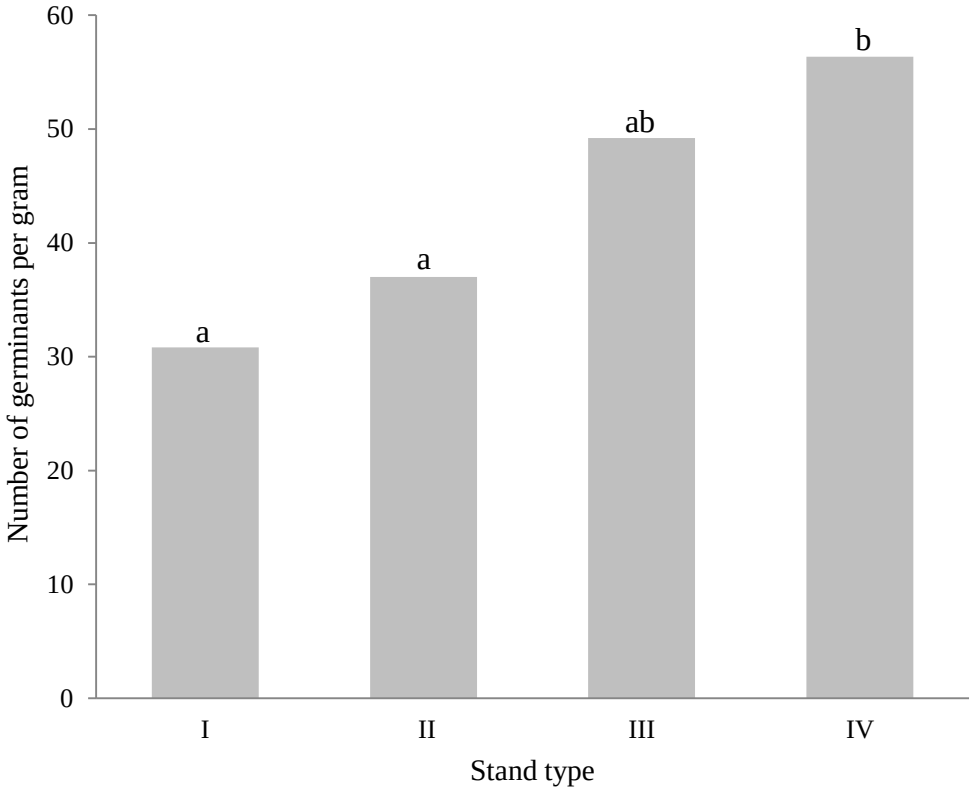

Fig. 2.2. Number of germinants in different stand types based on the 37 studied populations of *Eucalyptus pauciflora*. Stand type I and II were significantly different from stand type IV at P < 0.05.

Population differences in seedling survival in the nursery ranged from 83% to 99.5 % (mean = 94%, sd = 3.28, n = 37) and, across all replicates in the field trial, survival ranged from 93.7% to 100% (mean = 98%, sd = 1.67, n = 37). Neither survival rates were significantly different among populations (nursery - Kruskal-Wallis χ^2_{36} = 44, P =

0.165; field trial - Kruskal-Wallis $\chi^2_{36} = 36$, $P = 0.468$). Survival was not affected by the population altitude (nursery - $F_{1, 35} = 0.44$, $r^2 = 1$ %, $P = 0.508$; field - $F_{1, 35} = 0.29$, $r^2 = 0$ %, P = 0.590) nor the Stand Type (nursery - Kruskal-Wallis $\chi^2_3 = 7.43$, $P = 0.059$; field - Kruskal-Wallis $\chi^2_3 = 2.11$, $P = 0.549$). There was no significant correlation between population-level mating system estimates and seedling survival in the nursery or in the field.

2.4 Discussion

The overall multilocus outcrossing rate (t_m) obtained for *E. pauciflora* was high (90%), but comparable to that obtained in microsatellite studies of many other *Eucalyptus* species (*E. camaldulensis*: Butcher and Williams 2002; *E. morrisbyi*: Jones *et al.* 2005; *E. melliodora*: Broadhurst 2013; *E. melliodora*: Broadhurst 2013, *E. incrassate*: Breed *et al.* 2012). Our estimate was greater than the average allozyme-based outcrossing rate reported for 23 eucalypt species of 0.74 (Byrne 2008b), as well as that reported in a previous study of *E. pauciflora* (Phillips and Brown 1977). This previous study of three mainland *E. pauciflora* populations reported an allozyme-based multilocus outcrossing rate of 63% at the seed stage. This rate increased to 76% in two lower altitude populations when assessed in 6-week old seedlings, but did not reach the levels found in the present study in 1 year-old seedlings (8 months in the nursery after sowing and 4 months in the field). Our higher outcrossing rate estimate could be due to difference in assessment age, with selfed progenies having been eliminated from our older samples. The study of Phillips and Brown (1977) provided evidence of early-age post-zygotic selection against the products of self-fertilization (at least, in two of the three populations studied). There is also evidence for strong selection against the products of self-fertilization at later ages in other eucalypt species (Hardner and Potts 1997), as well as reports of rare deleterious mutations expressed in young seedlings (Patterson *et al.* 2000). However, there are other studies in which no evidence was found of early age inbreeding depression affecting germination or survival in the first year of field planting (Hardner and Potts 1995; Hardner and Potts 1997). In the present study, there was little evidence for early-age selection operating differentially among populations from the period between germination and sampling. Populations with high outcrossing rates also had high germinant yields per weight of capsule content; which is the opposite of what would be expected if high outcrossing levels were a consequence of early post-zygotic

selection against the products of self-fertilisation (Pound *et al.* 2003). Furthermore, overall mortality rates in the nursery and in the field trial (at the time of assessment) were low; population-level mortality was not correlated with outcrossing rate as might be expected if significant post-planting mortality of selfs as a driver of outcrossing rate variation. The most likely cause of the difference between the two studies of *E. pauciflora* is the use of different marker technologies. Allozyme markers can underestimate the true outcrossing rate by approximately 10 % (Byrne 2008b). This adjustment would bring the previous estimates for lower altitude populations close to our estimate. The possibility that the island and mainland populations have different inherent outcrossing rates cannot be completely dismissed. However, the differences between studies is the reverse of that which might be expected because, if anything, small, isolated island populations are more likely to have higher self-compatibility (and, thus, lower outcrossing rates) than more extensive mainland populations (Schueller 2004).

The significant bi-parental inbreeding (t_m-t_s = 0.16) observed in *E. pauciflora* is consistent with nearest-neighbour pollinations coupled with the presence of related individuals growing in close proximity in the mature forest. Such spatial clustering of related individuals in eucalypt forests is common, as a result of limited seed dispersal (Eldridge *et al.* 1993; Jones *et al.* 2007; Skabo *et al.* 1998). With such spatial structure, bi-parental inbreeding is favoured not only by synchronous flowering of related neighbours (flowering time is under strong genetic control in eucalypts: Jones *et al.* 2011) but also by nearest-neighbour foraging behaviour of animal pollinators, particularly insects (Morgan and Barrett 1990; Patterson *et al.* 2004). The bi-parental inbreeding observed in *E. pauciflora* is in the upper range of microsatellite-derived estimates from many eucalypt species, including *E. gomphocephala* (Bradbury and Krauss 2013), *E. melliodora* (Broadhurst 2013), *E. morrisbyi* (Jones *et al.* 2005) and *E. globulus* (Mimura et al. 2009), and is similar to species such as *E. incrassata* (Breed et al. 2012b) and *E. benthamii* (Butcher et al. 2005). The level of correlated paternity in *E. pauciflora* (r_p = 0.20) was also similar to that reported in other Australian plant taxa reviewed in, Coates *et al.* 2007, including *Eucalyptus* (Breed *et al.* 2012b; Broadhurst 2013; Butcher *et al.* 2005; Jones *et al.* 2005; Mimura *et al.* 2009). Some level of

correlated paternity would be expected simply through a tendency for nearest-neighbour matings, as discussed above.

There are few studies where the differences in mating system parameters among populations have been assessed in *Eucalyptus* (Bradbury and Krauss 2013; Mimura *et al.* 2009; Phillips and Brown 1977), and the present study is clearly the largest. An important finding of our study was that the significant variation in outcrossing rates among populations was related neither to stand fragmentation nor to altitude, as initially hypothesised. Population variation in mating system parameters may result from environmental and/or genetic effects (Levin 2012), and both may be involved in the variation in mating system among our wild-sampled trees. Environmental factors may include variation in pollinator availability or mobility (Groom 1998; Llorens *et al.* 2012; Wilcock and Neiland 2002) and plastic changes in floral biology (Kay and Picklum 2013), including self-incompatibility (McGowen *et al.* 2010). Indeed, several studies have shown that variation in outcrossing rate is linked to variation in self-incompatibility (Patterson *et al.* 2004; Willi and Määttänen 2010) which, in the case of the eucalypts, may have both genetic and plastic components (McGowen *et al.* 2010).

The earlier study of population variation in outcrossing rate in *E. pauciflora* suggested that outcrossing rate was inversely correlated with altitude (Phillips and Brown 1977). This trend may be expected due to severe environmental conditions at high altitude, such as low temperatures and strong winds, that limit pollen production or availability and reduce pollinator efficiency (Garcia-Camacho and Totland 2009). Such altitude-related factors may favour self-compatibility, thereby lowering outcrossing rates at higher altitudes, but evidence for this is equivocal (Arroyo *et al.* 2006; Wirth *et al.* 2010). A meta analysis by Garcia-Camacho and Totland (2009) failed to support this as a general trend and found cases where limited pollen availability at high altitude was compensated by higher pollinator efficiency (Arroyo *et al.* 2006), despite pollinator limitations (Totland 1993). Seasonal (year to year) variation in, for example, pollinator or flower abundance, may have contributed to the differences among populations observed by Philips and Brown (1977), but high stability of outcrossing rates across seasons has been reported within populations of *E. globulus* (McGowen *et al.* 2004). It is possible that the absence of an altitudinal effect on outcrossing rate in our study is

due to our upper altitudinal populations not being at the tree line as was the case for the upper population studied by Philips and Brown (1977) on mainland Australia. *Eucalyptus pauciflora* does not form the upper altitudinal tree line on Tasmanian Mountains whereas it does on the mainland. In the present study, the difference in altitude among populations ranged to more than 1100 m, while in the study of Philips and Brown (1977) the altitude differential was 340 m. Thus, if there were a purely altitudinal effect we expect that we would have found it. It is possible that the effect found by Philips and Brown (1977) was an edge effect at the tree line (Tarazi *et al.* 2013). For example, Mimura *et al.* (2009) noted that coastal populations of *E. globulus* had lower gene flow and hypothesised that this was due to the absence of forest on the seaward side.

While we found no effect of altitude on the outcrossing rate of *E. pauciflora*, correlated paternity did decrease with increasing altitude. Several factors may contribute to this decrease: greater pollen availability (Surles *et al.* 1990), greater pollen dispersal (Smouse *et al.* 1999), more synchronous flowering (Erickson and Adams 1989), and higher population density (Robledo-Arnuncio *et al.* 2004) at higher altitudes. *E. pauciflora* tends to flower in early spring at low altitudes and in mid-summer at higher altitudes (Duncan 1989; Pryor 1976). This later flowering may occur when ambient temperatures are higher which could lead to more synchronous flowering and a greater build up of insect pollinator diversity, abundance and activity, leading to greater gene flow and decreased correlated paternity. There is little published literature on the pollination biology of *E. pauciflora* but, as with most eucalypts with small flowers, it is probably pollinated by a diverse range of insects and less frequently by birds and mammals (House 1997). It is also possible that the difference in correlated paternity could reflect a change in the profile of the pollinator community with altitude which has been reported to occur in Tasmania (Hingston and McQuillan 2000). Bi-parental inbreeding decreased with altitude, but only in the more fragmented populations. This resulted in a trend for fragmentation to increase bi-parental inbreeding, but only at lower altitudes. Increased bi-parental inbreeding in the fragmented populations have been reported in other species (Bradbury and Krauss 2013; Breed *et al.* 2012a; Mimura *et al.* 2009), which might be due to the increased likelihood of mates being spatially proximal relatives (Sebbenn *et al.* 2011).

The degree of forest fragmentation does not appear to have a significant impact on the outcrossing rate in *E. pauciflora*. In many taxa, habitat disturbance, reduced stand density and increased population isolation, disrupts gene flow, resulting in increased inbreeding and reduced pollen diversity in forest fragments (Eckert *et al.* 2010; Kramer *et al.* 2008; Lowe *et al.* 2005; Sork and Smouse 2006). While a negative effect of fragmentation on outcrossing rate has been reported in some *Eucalyptus* species (Butcher *et al.* 2005; Hardner *et al.* 1996; Millar *et al.* 2000; Mimura *et al.* 2009), several recent studies have found limited or no impact of forest fragmentation on the mating system parameters (*E. incrassata*: Breed et al 2012; *E. melliodora*: Broadhurst 2013; *E. gomphocephala*: Bradbury and Krauss 2013). In some species, even small, isolated, undisturbed populations appear to maintain high outcrossing rates through strong self-incompatibility mechanisms (*E. morrisbyi*: Jones *et al.* 2005). Late-acting post-zygotic self-incompatibility mechanisms are common among the eucalypts (Horsley and Johnson 2007; Pound *et al.* 2002) and may help to buffer a population against the expected negative consequences of forest fragmentation (Byrne *et al.* 2008; Kramer *et al.* 2008).

The fact that forest fragmentation reduced the number of germinants per gram by up to 45% is consistent with a strong late-acting post-zygotic self-incompatibility mechanisms operating in *E. pauciflora*. The absence of an effect of fragmentation on mating system, but an impact on seed yield, has also been reported in other taxa (Friedman and Barrett 2008), including eucalypts (Broadhurst 2013; Burrows 2000; Krauss *et al.* 2007). Burrows (2000) reported a 60% reduction in seed germinant yield when comparing woodland trees to isolated trees of *E. melliodora*, somewhat higher than our 45% lower yield in isolated trees. In some eucalypt species (e.g., *E. globulus*: Mimura *et al.* 2009; *E. benthamii:* Butcher *et al.* 2005) a reduction of both outcrossing rate and seed germinant yield with fragmentation has been reported, while in *E. gomphocephala* there was little or no impact of fragmentation on either outcrossing rate or germinant yield (Bradbury and Krauss 2013). The impact of fragmentation on outcrossing rate and germinant yield may depend on the strength and nature of the self-incompatibility mechanism(s) of the species involved. In *E. pauciflora*, the reduced germinant yield in the presence of high outcrossing could be a result of a strong post-

zygotic self-incompatibility mechanism coupled with either (i) reduced number of pollinations through reduced pollinator activity in fragmented populations the 'Allee effect', Groom 1998; Wilcock and Neiland 2002; see also Aguilar *et al.* 2008; Quesada *et al.* 2013), or (ii) an increased self-fertilization rate in fragmented populations followed by post-zygotic abortion of the selfed seed (Horsley and Johnson 2007; Pound *et al.* 2002).

The variation in outcrossing rates observed among populations of *E. pauciflora* may be due to other factors not assessed in this study. The mating system may be influenced by the position of a population relative to the geographic distribution of the species (Michalski and Durka 2007; Tarazi *et al.* 2013). Peripheral populations may be exposed to different abiotic and biotic factors, subject to greater fluctuations in population size and density and be more prone to founder effects which favour selfing as a reproductive assurance strategy (Leimu *et al.* 2006; Michalski and Durka 2007). Populations occupying recently colonised areas often show increased self-compatibility, a phenomenon known as "Bakers Law" (Baker 1955; Cheptou 2012). It appears, however, that populations of Tasmanian *E. pauciflora* do not conform to Baker's Law: populations that have the lowest outcrossing rates tend to occupy refugial areas rather than areas of more recent post-glacial recolonisation (Williams 1991). Environmental stresses such as drought or salinity can change pollinator activity, phenology and physiology of plants that can drive a transition to increased self-compatibility (Kay and Picklum 2013). Plants are expected to become more stressed with global climate change (Beaumont *et al.* 2011), and this is likely to be expressed most strongly at the trailing edge of species distributions (Levin 2012). Indeed, Levin argues that plastic changes in the mating system in these populations may lead to increases in self-compatibility and selfing rates as an adaptive mechanism in stressed environment (Levin 2012). Tree decline and dieback have been particularly severe over the last decade in Tasmania (Close and Davidson 2002), partly due to severe drought periods (Jurskis 2005; Neyland 1996). Such stresses may have caused population differences in the mating system of *E. pauciflora*. However, the focal point of severe tree decline is mainly in the midland regions of Tasmania and does not correspond well with the distribution of populations with lower outcrossing rates, and regardless there was also no effect of fragmentation.

In conclusion, outcrossing rates were variable among populations of *Eucalyptus pauciflora*, although no populations had low outcrossing rates. The variation in outcrossing rate was not related to variation in the degree of fragmentation nor altitude of the population. While fragmentation may increase bi-parental inbreeding, this trend was only evident at lower altitudes. With this exception, these results argue that in most cases restoration plantings established from seed collected from fragmented forests are unlikely to experience more inbreeding depression than those established using seeds collected from continuous forests. This resilience to habitat disturbance might be due to strong genetic-based self-incompatibility in *E. pauciflora*. The reduced seed yield from capsules collected from fragmented populations can be countered by collection of more seed per tree or, if capsule crops are limited, by collection of seed from more trees. While most populations are highly outcrossed, the extent to which the reduced outcrossing in particular populations is a stable or transitory effect of the pollinator environment, or a plastic or genetic attribute of the tree, requires further study.

2.5 Acknowledgements

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Chapter 3. Molecular genetic diversity and population structure in *Eucalyptus pauciflora*

Abstract

Genetic diversity and population structure in *Eucalyptus pauciflora* was assessed using seven chloroplast microsatellite markers and 10 nuclear microsatellite markers. Thirty-seven populations (281 wild trees) were sampled across the species' geographic and altitudinal distribution in Tasmania. In addition, samples were collected from five to eight open-pollinated progenies from each tree giving a total of 1,359 samples. Thirty-one chloroplast haplotypes were identified from the wild trees. The distribution of chloroplast haplotype richness showed a clear geographic structure with suggestion of three major refugia (Storm Bay, Tamar Valley and St Pauls River Valley) two of which are consistent with previously reported glacial refugia for other eucalypts. Chloroplast haplotype affinities provided evidence of migration of populations from north and east towards the south and west of Tasmania. High nuclear microsatellite diversity was observed across the species' range in both maternal samples and progenies. Most of this variation was distributed within populations with low but significant F_{ST} (maternal samples = 0.034; progenies = 0.055), suggesting high gene flow among populations that is more manifest in the mature stand. Higher nuclear genetic diversity in newly colonized areas compared to lowland putative refugial regions, and the converse in chloroplast DNA markers, suggest limited seed dispersal into newly colonised regions combined with high pollen flow between different source populations in newly colonised areas. Our results provide evidence against the hypothesis that highland populations of *E. pauciflora* originate from *in situ* high altitude refugia but instead originate from lowland refugia.

Keyword: *Eucalyptus pauciflora*, molecular markers, haplotypes, genetic diversity, spatial structure, glacial refugia

3.1 Introduction

Eucalyptus pauciflora has a widespread distribution throughout the south east of Australia (28°S to 42.5°S), forming the tree-line on most of the mountains of the Australian Alps and extending to near sea level in Southern Victoria and Tasmania (Boland *et al.* 2002; Williams 1991; Williams and Potts 1996). In Tasmania, *E. pauciflora* subsp. *pauciflora* (hereafter as abbreviated to *E. pauciflora*) is the only naturally occurring subspecies, growing from 10 m to 1080 m above sea level (Boland *et al.* 2002; Williams and Ladiges 1985). It is a dominant species of many of the forests and woodlands in cold, dry regions of the central and eastern part of the island (Williams and Potts 1996) and is replaced at the tree-line by *E. coccifera*. *Eucalyptus pauciflora* also has a relatively wide climatic envelope: for example, mean annual temperatures range from 4.1°C to 15.4°C and mean annual precipitation varies from 450 mm to 2537 mm (Williams 1991).

Southern Victoria on continental Australia and the island of Tasmania share many plant species, including *E. pauciflora* (Nicolle 2006b). This is probably because of the repeated formation of land bridges between Victoria and Tasmania during Quaternary/Pleistocene glacial periods when sea levels were much lower (Kirkpatrick and Fowler 1998; McKinnon *et al.* 2004a; McKinnon *et al.* 1999; Steane *et al.* 1998). There is geological evidence for at least five glacial periods in Tasmania, and during interglacial periods Tasmania and the mainland would have become isolated. During glacial periods gene flow may have occurred across Bass Strait; the presence of remnant coastal populations of *E. pauciflora* on either side of Bass Strait indicate the possibility that the Bassian Plains were forested with this species (Kirkpatrick and Fowler 1998). Morphological (Williams and Ladiges 1985) and pollen studies (Dodson 1977) have suggested that the present-day low altitude populations of *E. pauciflora* are relicts from the most recent glacial period. Expansion of these refugial populations by upslope range migration as the climate became warmer might have resulted in the current high altitude populations (Dodson 1977; Williams and Ladiges 1985). However, Hope and Kirkpatrick (1989) proposed that the highland populations might have resulted from expansion out of high altitude refugia where *E. pauciflora* might have been present as rare savannah trees within a grassy/daisy steppe. Both hypotheses on the origin of high altitude populations were given support in a climate modelling study of *E. pauciflora*

(Williams 1991), as this would explain how *E. pauciflora* managed to occupy most of its predicted range.

On the basis of its broad ecological tolerances and successful common garden field trials (Close and Davidson 2002; Close *et al.* 2010), *E. pauciflora* has been selected as a key species for ecological restoration and carbon planting in Tasmania (Bailey *et al.* 2013). Thus, a better understanding of this species, including its genetic architecture and the evolutionary processes shaping the patterns of genetic variation is important. Understanding the evolutionary processes operating in current and past environments requires a detailed knowledge of species' genetic diversity and gene flow mechanisms (Neale and Kremer 2011; White *et al.* 2007). Molecular methods are standard techniques used to investigate processes of species evolution and population dynamics (Duran *et al.* 2009; Steane *et al.* 1999). Chloroplast and nuclear markers, when used together, are powerful tools for understanding historical and contemporary processes that have contributed to the present day gene pool. Chloroplast DNA (cpDNA) is used widely for phylogeographic studies because of its uniparental mode of inheritance (maternal in most angiosperms), absence of recombination and low rate of mutation mean that the molecule retains ancient patterns of genetic diversity and can be used to infer historical processes such as refugial isolation and post-glacial recolonisation (Petit *et al.* 2005). Chloroplast DNA markers have been used widely for the analysis of postglacial recolonisation (Kremer *et al.* 2010; Okaura *et al.* 2007; Petit *et al.* 1997; Worth 2009), and studies have included several species of eucalypt (Bloomfield *et al.* 2011a; Byrne 2008a; Freeman *et al.* 2001; McKinnon *et al.* 2004a; Nevill *et al.* 2010). The historical information gained from cpDNA can be combined with information from biparentally-inherited, rapidly evolving nuclear DNA (e.g, nuclear microsatellites (SSRs)) to assess spatial genetic structure, diversity and gene flow.

In this study, we use both nuclear and chloroplast microsatellite markers to examine the spatial distribution of genetic diversity in *E. pauciflora* in Tasmania. We aim to test four hypotheses: i) there is little neutral molecular genetic differentiation amongst populations in Tasmania; ii) spatial distribution of cpDNA will provide evidence of the historical gene flow of the species; iii) whether high land populations of *E. pauciflora* are derived from lowland refugia or from highland refugia; and iv) contemporary gene

flow, depicted through nuclear markers, will be different from the historical gene flow of the species shown by chloroplast markers. Information on genetic diversity, gene flow, migration and dispersal of the species from these potentially neutral markers will inform quantitative genetic studies of the species as well as guide seed collection and restoration decisions.

3.2 Materials and methods

3.2.1 Sample collection and DNA extraction

Leaf samples and seed capsules derived from open-pollination were collected from five to eight mature trees from each of 37 native populations across the entire geographic and ecological distribution of *E. pauciflora* in Tasmania (see Table 2.1), giving a total of 281 Tasmanian wild samples. Trees were sampled in such a way that the distance between two consecutive trees was at least double the tree height, to avoid sampling within the family group structure known to occur in eucalypts (Jones *et al.* 2007; Skabo *et al.* 1998). Altitude and the geographic coordinates of each sampled tree were recorded. The open-pollinated seeds were used to establish several progeny trials, with each family represented by a single tree in each replicate (Bailey *et al.* 2013). When the seedlings were one year old, leaf material was collected from a field trial at Dungrove (42° 16' 29.3052" S, 146° 53' 28.0098" E) in such a way that 40 seedlings were sampled per population. These seedlings were taken from five to eight families per population so that, given 37 populations, a total of 1,480 seedlings were sampled (see Chapter 2). At the time of sampling, seedling mortality rate was 2%, and there was no significant difference in field mortality rate among populations (see Chapter 2). Leaves collected from the maternal samples were dried in silica gel, but leaves from seedlings were freeze-dried and sealed in aluminium foil bags until DNA extraction.

3.2.2 Molecular methods

Genomic DNA was extracted from leaves using a modified CTAB method (Doyle and Doyle 1990; McKinnon *et al.* 2004b). Seven chloroplast microsatellite primer pairs (EMCRC59cp, EMCRC60cp, EMCRC65cp, EMCRC67cp, EMCRC74cp, EMCRC86cp and EMCRC90cp; Steane *et al.* 2005) were tested in 16 randomly selected samples (including mainland samples supplied by Dr Michael Bayly, University of Melbourne, Australia). Five microsatellite primer pairs namely

EMCRC60cp, EMCRC67cp, EMCRC74cp, EMCRC86cp and EMCRC90cp were previously used in previous studies on related *Eucalyptus* species (Bloomfield *et al.* 2011a; Nevill 2010; Nevill *et al.* 2010). While all microsatellites were polymorphic in mainland populations, only primer pairs EMCRC59cp, EMCRC60cp, EMCRC65cp and EMCRC86cp were polymorphic in Tasmania. In order to make results comparable with mainland populations and previous studies, all primer pairs were used in this study.

In addition, 15 nuclear microsatellites (CRC6, CRC11 (Steane *et al.* 2001), EL13 (Ottewell *et al.* 2005), ES140, ES157, ES211, ES255 (Glaubitz *et al.* 2001), EMBRA08, EMBRA011, EMBRA042, EMBRA187, EMBRA196, EMBRA210, EMBRA232 (Brondani *et al.* 1998; Brondani *et al.* 2006), and EPIL_MYB2 (Shepherd *et al.* 2010)) were screened against the same 16 individuals. Of these, two loci (EMBRA042 and EPIL_MYB2) did not amplify and, hence, 13 were used for further analysis. Studied loci and their characteristics are shown in Table 3.1.

Forward primers of each chloroplast and nuclear locus were labelled with NED, VIC, 6-FAM, or PET fluorescent dye (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) for chloroplast microsatellite loci was performed in 12.5 µl reactions containing approximately 1 µl of 20 ng DNA, 6.25 µl of 2X QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$), and 1.25 µl of primer mix containing 10 µM of each forward and reverse primer. PCR for nuclear microsatellite loci was performed in 5 µl reactions containing approximately 1 µl of 20 ng DNA, 2.5 µl of 2X QIAGEN Multiplex PCR Master Mix (providing a final concentration of 3 mM $MgCl_2$), 0.5 µl of 5X Q-Solution, and 0.1 µl of primer mix containing 10 µM of each forward and reverse primer.

The PCR profile consisted of 15 min denaturation at 95 °C followed by 30 cycles of 94 °C for 30 sec, 90 sec at an annealing temperature of 53 °C to 60 °C (depending upon annealing temperature of each primer pair used; Table 3.1) and 72 °C extension for 60 sec, followed by a final extension of 10 min at 60 °C. PCR products were checked for quality, diluted and combined as needed and were sent to the Australian Genome Research Facility Ltd. (Adelaide) for capillary separation on an ABI3730 analyser

(Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Alleles were sized and scored using Genemapper software version 3.7 (Perkin Elmer, Applied Biosystems).

Samples that were missing data for five or more nuclear loci were discarded from the analysis, reducing the sample size to 276 maternal samples and 1,359 progenies. In the chloroplast DNA set, one sample could not be amplified, so a total of 280 samples were included in the final analysis.

Maternal samples were unavailable for the South Arm population. To overcome this problem, MLTR version 3.1 (Ritland 2002) was used to infer the most likely maternal nuclear genotypes, using data from the progeny array. These inferred 'maternal' genotypes were used in subsequent analyses.

3.2.3 Chloroplast microsatellite analysis

Within populations, the number of chloroplast haplotypes (*A*), number of private chloroplast haplotypes (A_E), and chloroplast haplotype composition for each population was calculated using GENALEX 6.501 (Peakall and Smouse 2006). Haplotypes are defined as a distinct combination of the alleles at a given set of microsatellites. Haplotypes were classified and named according to Nevill *et al*. (2010) and Bloomfield *et al*. (2011b). However, as the current study used two more markers than these previous studies, each haplotype was named as an extension to previously described ones. For example, haplotypes 19/1 and 19/2 in this study are identical in the five microsatellites of haplotype 19 of previous studies, but with additional information from two more microsatellite markers. New haplotypes were named in the order of discovery, following those already listed for *E. obliqua*, *E. delegatensis* and *E. regnans*. Haplotypes that were in only one population were defined as 'private' haplotypes. As the current study had an uneven sample size, rarefied haplotype richness (*R)* was computed using Contrib 1.02 (Petit *et al.* 1998).

Table 3.1. Nuclear microsatellite loci used to study Tasmanian *Eucalyptus pauciflora*. Repeat motif; forward (F) and reverse (R) primer sequence (5´-3´); product size range (*PSR*); annealing temperature (°C); position of each SSR locus in *E. globulus* linkage map; dye used for labelling forward (F) primer.

SSR locus	Repeat motif	Primer sequences 5'-3´	*PSR*	Annealing temp. (°C)	Linkage group, position (cM); data source	dye
CRC11	$(TC)_{10}(AC)_{10}$	F: AACTGACTGTGGATTTGAAGC R: GTGAGTCATTATTTGGCAACC	216-268	60	6, 50.1; Hudson 2012	VIC
EL13	$(TC)_{17}(AC)_{10}$	F: CAAGAGTCACAGCCAAGCC R: GACAACGCATCTTTCTTTCTG	160-184	60	10; (1966423.1966584 bp); this study[1]	FAM
EMBRA011	$(AG)_4GG(AG)_{13}$	F: GCTTAGAATTTGCCTAAACC R: AGGATTTGTGGGGCAAGT	105-165	53	1, start of chromosome; Hudson 2012	FAM
EMBRA187	$(GA)_9CAGG(GA)_{20}$	F: CTCATGCATAGCTGCTACTC R: GCAGCTCAGTGTACATTGG	176-220	53	6, 19.9; Hudson 2012	FAM
EMBRA196	$(GA)_{46}$	F: GTGAAGCTCAACCTGTTGTCT R: GTGACCGATCATGTGTGGACT	243-349	57	6, 39.4; Hudson 2012	FAM
EMBRA210	$(TC)_{25}$	F: CGTGTGGTTATGTGAACT R: CCTAACAATGCATAAGCTC	190-236	53	9, 70.2; Hudson 2012	NED
EMBRA232	$(AG)_{12}$	F: TCCTTATCGTCAATTCTTGC R: GGTCTAGCGTGATTCATCCT	102-160	55	4; Brondani *et al.* 2006	PET
ES140	$(GT)_{20}(GA)_{10}$	F: GCTCATTGTACTGCACAGAGG R: AAGGCACCAACAGTACCTGG	122-180	60	9, 45.6; Hudson 2012	VIC
ES211	$(GA)_{17}$	F: GGGAGAGCTGATTGAGTAATTG R: GCTGAGAATGGAAGCACATC	84-118	60	9, 55; (Mapped to LG9 in Evandro's map (unpublished), 6.5 cM from EMBRA18. In composite map, EMBRA18 mapped to LG9, 49.13)	FAM
ES255	$(GT)_{12}$	F: TTTGCCATAGCGAAGTGTTG R: GACCACTTACCAAACTCACCG	91-107	60	(Not mapped by Thumma 2010, primers do not return blast hits in *E. grandis* sequence (BOGAS server))	PET

1 Blasted to *E. grandis* genome
(Phytosome: http://www.phytozome.com/search.php?show=blast&targetType=genome&method=Org_Egrandis); position is in bp (not cM).

The program Permut (available at http://www.pierroton.inra.fr/genetics/labo/Software /PermutCpSSR/index.html) was used to compute (i) the mean within-population genetic diversity, (ii) species-level total genetic diversity, and (iii) population differentiation calculated with alleles treated as unordered, where comparisons do not account for variation in allele size (h_S, h_T and G_{ST} respectively), and ordered, where the assumed number of mutational steps between alleles provides additional information (v_S, v_T and N_{ST} respectively). Population differentiation parameters, G_{ST} and N_{ST} were used to test for phylogeographic structure. When N_{ST} was significantly higher than G_{ST} we inferred that closely related haplotypes are more likely to occur within a population than less closely related haplotypes, indicating a degree of phylogeographic structure (Pons and Petit 1996). A haplotype network was constructed using the medium joining network algorithm (Bandelt *et al.* 1999) using Network 4.6.1.1 (fluxus-engineering 2012) to visualize the number of base pair differences between haplotypes. The difference between each pair of haplotypes was the sum of nucleotide differences between them over the four polymorphic chloroplast loci. Analysis of molecular variance (AMOVA) was performed on the allelic data to estimate the partitioning of genetic variation within and among populations. AMOVA was computed using GENALEX 6.501 (Peakall and Smouse 2006) and significance testing was performed using 9,999 permutations.

3.2.4 Nuclear microsatellite analysis

Population genetic parameters were estimated using various software packages. The number of alleles (*A*), observed (H_o) and expected heterozygosity (H_e) and Wright's fixation index (*F*) were calculated and averaged over loci and populations using GDA 1.1 (Lewis and Zaykin 2002). Null allele frequencies ($A(0)$) in each locus were estimated using GENEPOP (Rousset 2008). F-statistics (F_{IS}, F_{IT} and F_{ST}, Weir and Cockerham 1984) for each locus, and pairwise F_{ST} values among populations were estimated using FSTAT 2.9.3 (Goudet 2001). F-statistics were calculated with 99% confidence intervals using 1000 bootstrap replicates. FSTAT 2.9.3 (Goudet 2001) was used to calculate the Rarefied Allelic Richness (A_R; El Mousadik and Petit 1996) for each population, using a minimum sample size of four.

A pair-wise matrix of Nei's genetic distance was calculated using GENALEX 6.501 (Peakall and Smouse 2006). The significance of the association between genetic

distance and the geographic distance was tested using a Mantel test (Mantel 1967) with 10,000 permutations. Spatial autocorrelation analysis was used to determine the pattern of change of genetic distance with reference to geographic distance. The Mantel test and spatial autocorrelation analysis were performed in GENALEX 6.501. The relationships among populations were explored through UPGMA clustering, using Nei's genetic distance (Nei 1972). UPGMA dendrograms were constructed from 1,000 bootstrap replicates using GDA 1.1 (Lewis and Zaykin 2002) software and figures were produced using software TREEVIEW (Page 1996). To further study relationships among individuals and populations, structure analyses, based on a Bayesian clustering approach, was performed using STRUCTURE 2.3 (Pritchard *et al.* 2000). Assuming no prior population grouping, and using the options of admixture and both correlated and uncorrelated allele frequencies, the optimum value of the number of groups of genetically similar individuals (K) was determined. For the analyses, 100,000 Markov chain Monte Carlo repetitions, after a burnin period of 100,000 iterations, was used. The optimum value of K was determined from five runs at each value of K, ranging from K = 1 to K = 20, using the method described by Evanno *et al.* (2005) and the *ad hoc* statistic ΔK, based on the rate of change in the log probability of the data between successive K values. Structure Harvester v 0.6.93 (Earl and vonHoldt 2012) was used to choose the K value that best fit the data, using both log posterior probability of the data (ln Pr (X/K)) and ΔK (Evanno *et al.* 2005) .

Genetic differentiation estimates from nuclear markers (nssr) and chloroplast markers (cpssr) were used to estimate the relative influences of pollen- and seed-mediated gene flow in *E. pauciflora*, using equation 5a of Ennos (1994):

Pollen flow/seed flow = $\left[\left(1/G_{ST(nssr)}-1\right)(1+F_{IS})-2\left(1/G_{ST(cpssr)}-1\right)\right]/\left(1/G_{ST(cpssr)}-1\right)$

3.3 Results

3.3.1 Chloroplast haplotype diversity

Four of the seven chloroplast microsatellites (EMCRC59cp, EMCRC60cp, EMCRC65cp and EMCRC86cp) were polymorphic in Tasmanian *E. pauciflora*, yielding three, five, five and four alleles, respectively. Allelic variation at the four loci combined into 31 haplotypes (Table 3.2). The haplotype tree (Fig. 3.1) showed a relatively continuous network of haplotypes with most differentiated by only one base-

pair from adjacent haplotypes. A few haplotypes were separated by longer distances resulting from multiple changes at a single locus: in H56 there was a change of three base pairs at EMCRC60, and a cluster of haplotypes (H61, H60, H43/1 and H24/1) was separated from the main network by 8 base-pair differences at EMCRC65.

Half of the haplotypes were present at very low frequencies (≤ 0.7%; Table 3.2). H24/3 was the most frequent haplotype (present at a frequency of 20.4%) followed by H21/3 (17.9%). Mean rarefied haplotype richness per population was 1.3 and twelve (45%) haplotypes were population-specific (see 'private haplotypes' in Table 3.3). Overall, there was moderate within-population diversity (h_S = 0.49±0.048, v_S = 0.45±0.053), and high total chloroplast diversity (h_T = 0.91±0.018, v_T = 0.91±0.023).

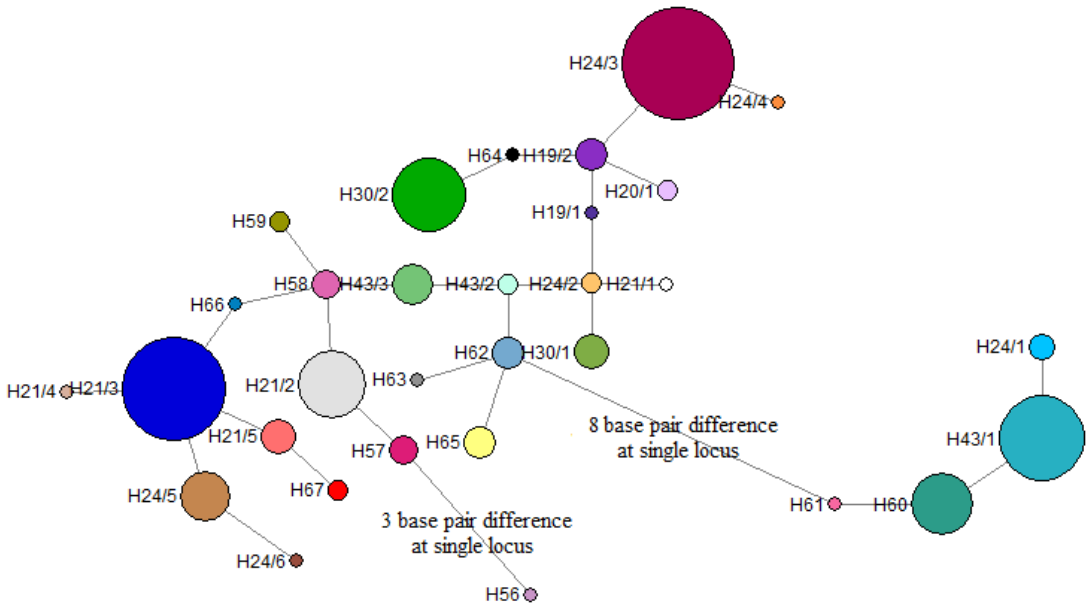


Fig. 3.1. Statistical parsimony tree of chloroplast microsatellite haplotypes found in the Tasmanian *Eucalyptus pauciflora*. The size of the circle represents the relative frequency of a haplotype. Each haplotype is represented by a unique colour. Branch lengths indicate the number of nucleotide differences between haplotypes. Branch lengths equal one base pair difference unless indicated.

Table 3.2. Chloroplast haplotypes found in *Eucalyptus pauciflora* in Tasmania, their respective frequencies and counts and the allele at each cpSSR locus. The presence of the haplotype class in other species is given as *E. obliqua* (obl), *E. delegatensis* (del), *E. regnans* (reg).

Haplotypes	Frequency (%)	Count	EMCRC59	EMCRC60	EMCRC65	EMCRC86	*Presence in other species
H19/1	0.4	1	241	195	254	147	obl, reg
H19/2	1.8	5	241	195	255	147	obl, reg
H20/1	0.7	2	241	194	255	147	del, obl, reg
H21/1	0.4	1	241	194	254	148	obl, reg
H21/2	7.5	21	241	194	255	148	obl, reg
H21/3	17.9	50	242	194	255	148	obl, reg
H21/4	0.4	1	242	194	256	148	obl, reg
H21/5	2.1	6	243	194	255	148	obl, reg
H24/1	1.1	3	241	195	245	148	del, obl, reg
H24/2	0.7	2	241	195	254	148	del, obl, reg
H24/3	20.4	57	241	195	255	148	del, obl, reg
H24/4	0.4	1	241	195	256	148	del, obl, reg
H24/5	3.9	11	242	195	255	148	del, obl, reg
H24/6	0.4	1	242	195	256	148	del, obl, reg
H30/1	2.1	6	241	196	254	148	obl, reg
H30/2	8.9	25	241	196	255	148	obl, reg
H43/1	11.8	33	241	195	245	149	del
H43/2	0.7	2	241	195	254	149	del
H43/3	2.9	8	241	195	255	149	del
H56	0.4	1	241	190	255	148	
H57	1.4	4	241	193	255	148	
H58	1.4	4	241	194	255	149	
H59	0.7	2	241	194	255	150	
H60	6.1	17	241	196	245	149	
H61	0.4	1	241	196	246	149	
H62	1.8	5	241	196	254	149	
H63	0.4	1	241	196	254	150	
H64	0.4	1	241	196	255	147	
H65	1.8	5	241	196	255	149	
H66	0.4	1	242	194	255	149	
H67	0.7	2	243	193	255	148	

In addition to the four loci shown here, three more (EMCRC67, EMCRC74 and EMCRC90) were also used for the study. These three loci were monomorphic in Tasmanian *E. pauciflora*; EMCRC67 was fixed at 234 bp, EMCRC74 at 127 bp and EMCRC90 at 231 bp.

*Presence of haplotype class (e.g., H19); for details see methods.

Populations were highly differentiated, with a G_{ST} of 0.47 ± 0.049, N_{ST} of 0.51 ± 0.056 and AMOVA indication that 88% of the variation was between populations ($P < 0.001$). Both G_{ST} and N_{ST} were significantly different from zero ($P < 0.001$), indicating significant genetic structure. N_{ST} was not significantly greater than G_{ST}, providing little support for clear phylogenetic structure of haplotypes among populations. There was a weak but significant signal of isolation by distance among populations (Mantel r = 0.12, $P = 0.044$). The spatial autocorrelation analysis indicated that genetic distance between populations increased linearly up to 93 km, with populations within 38 km being significantly more similar in their chloroplast microsatellite affinities than would be expected from chance (Fig. 3.2a). The spatial distribution of haplotypes in each population, showed geographical structure, especially in the central region of the *E. pauciflora* distribution, where each of three regions was dominated by a distinct haplotype (Fig. 3.3). The central west region was dominated by haplotype H21/3, the central region by H24/3 and the central east by H43/1. This dominance resulted in these haplotypes being the most common haplotypes in *E. pauciflora* (Fig. 3.1). H24/3 dominated mid-altitude (347 m to 895 m) populations on the south-eastern slopes of the Central Plateau of Tasmania (populations 17, 20, 25, 29, 30 & 31), but also occurred at low frequency in an adjacent western population (21) as well as in lower-altitude (16 m to 498 m) populations to the north (1, 3, 4, 5, 6, 8 and 11), including the near-sea-level population at Waterhouse (1) in the far north-east of the distribution. H21/3, together with phylogenetically similar haplotypes H24/5 and H66, completely dominated the high-altitude (460 m -1138 m) western populations (10, 13, 14, 18, 22, 26), but H21/3 also occurred in lower altitude populations in the southern (35 m to 100 m) and northern (3 m to 282 m) central regions. The other common haplotype, H43/1 and phylogenetically similar haplotypes (H60 and H24/1), dominated a wide altitudinal range in the southern Midlands and Eastern Tiers (229 m to 626 m), but these haplotypes also occurred in two highly polymorphic populations, one in the northern Midlands (9 m to 160 m) and the other to the south (28 m to 412 m).

Across the range of *E. pauciflora* in Tasmania, chloroplast haplotype diversity was not associated with altitude ($F_{1,35} = 0.01$, $r^2 = 0$ %, $P = 0.91$; Table 3.4), but populations formed three spatial clusters, representing 'hot-spots' of haplotype diversity (Fig. 3.4a). The first cluster comprised the low-altitude (16 m to 405 m) northern populations (1, 3,

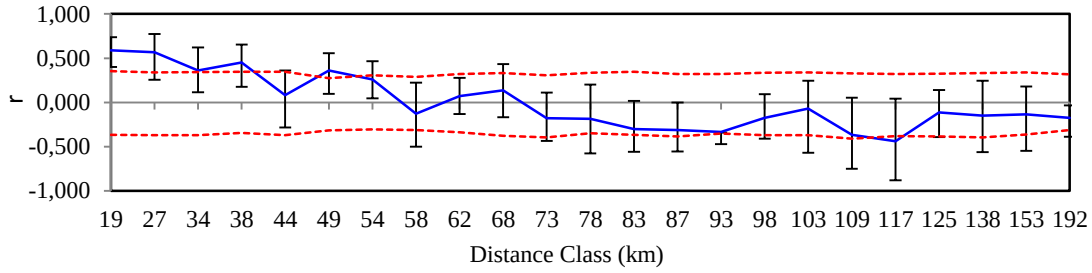
5, 9) extending from the northern Midlands to the north-east coast (Tamar Valley), the second comprised the low altitude (41 m to 444 m) populations in the south-east (Storm Bay region) (28, 32, 33, 34, and 36), and the third cluster included two eastern populations in the upper Avoca Valley (8) and Dukes Marsh (11) (St Pauls River Valley) between 237 m and 498 m altitude. While the most southern, small disjunct population growing near sea-level at South Arm (37) was relatively depauperate in chloroplast haplotype diversity, it contained the same haplotype (H24/2) as well as haplotypes that differed from H24/2 by only one mutation (H43/2, H30/1, H19/1 and H21/1; Fig. 3.1) as its two closest northern populations (33 and 36), suggesting an historic link. The populations in central Tasmania tended to have relatively low chloroplast haplotype diversity (Fig. 3.3).

A comparison of haplotype diversity of *E. pauciflora* with *E. obliqua* (Bloomfield *et al.* 2011b), *E. delegatensis* and *E. regnans* (Nevill 2010; Nevill *et al.* 2010), revealed five haplotypes (H19, H20, H21, H24, H30) that were shared with *E. obliqua* and *E. regnans*, and three haplotypes (H20, H24 and H43) that were shared with *E. delegatensis*. However, there was no geographic coincidence in the distribution of shared haplotypes across Tasmania for these species.

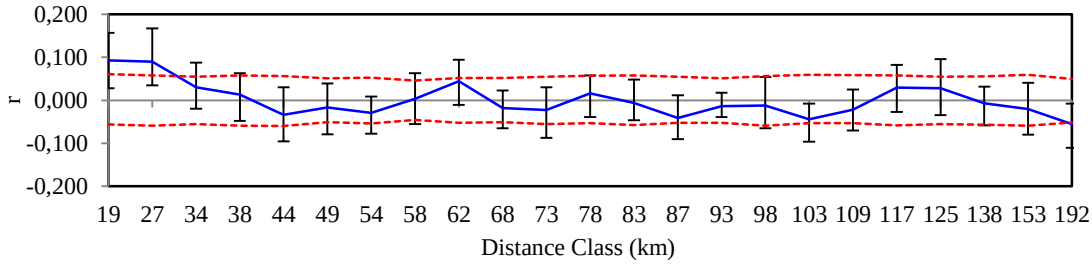
Table 3.3. Tasmanian *Eucalyptus pauciflora* populations studied with their codes, geographic locations, number of haplotypes (*A*), number of private haplotypes (A_E), rarefied haplotype richness (*R)* and haplotypes codes (when more than one sample shared a haplotype, the number is indicated preceding the symbol X). Private haplotypes are underlined.

ID	Population	Latitude (°S)	Longitude (°E)	Altitude	*A*	A_E	*R*	Haplotypes			
1	Waterhouse	-40.91	147.66	16	4	0	2.5	3×H24/3	H30/2	H43/3	H65
2	Nunamara	-41.37	147.32	405	3	1	1.5	5×H21/5	H43/3	2×H67	
3	Brushy Lagoon	-41.41	146.75	282	4	0	2.6	2×H21/2	2×H21/3	2×H24/3	H30/2
4	Tyne River	-41.47	147.82	297	2	0	1.0	2×H24/3	5×H30/2		
5	Longford	-41.63	147.10	159	4	0	2.3	4×H21/2	2×H24/3	2×H43/3	2×H58
6	Symmons Plains	-41.66	147.25	166	3	0	1.7	4×H21/2	H24/3	H43/3	
7	Rossarden	-41.69	147.70	731	2	0	0.7	H20/1	6×H21/2		
8	Avoca	-41.71	147.83	237	4	0	2.7	H21/5	H24/3	2×H43/3	2×H65
9	Cressy	-41.72	147.10	160	5	0	2.9	H20/1	2×H24/1	2×H43/1	2×H58
								H65			
10	Lake Rowallan	-41.72	146.22	460	2	0	0.6	7×H21/3	H24/5		
11	Dukes Marshes	-41.72	148.13	498	4	1	2.1	4×H19/2	2×H24/3	H43/3	H64
12	Conara	-41.84	147.46	206	2	1	1.0	4×H21/2	4×H57		
13	Lake Arthur	-41.96	146.88	1004	2	0	0.6	8×H21/3	H24/5		
14	Great Lake	-41.99	146.70	1138	2	0	1.0	4×H21/3	6×H24/5		
15	Ross	-42.00	147.53	240	1	0	0.0	9×H43/1			
16	Lake Leake	-42.02	147.82	597	2	0	1.0	4×H43/1	5×H60		
17	Wihareja	-42.06	146.81	895	2	0	1.0	5×H24/3	2×H30/2		
18	Pine Tier	-42.09	146.52	818	2	0	1.0	5×H21/3	3×H24/5		
19	Tunbridge	-42.12	147.36	229	3	0	1.1	H24/1	7×H43/1	H60	
20	Interlaken	-42.15	147.14	818	1	0	0.0	8×H24/3			
21	The Point	-42.19	146.42	674	3	0	1.4	H21/3	H24/3	5×H30/2	
22	Lake St Clair	-42.20	146.14	816	1	0	0.0	8×H21/3			
23	Woodbury Hill	-42.21	147.28	626	1	0	0.0	6×H43/1			
24	Tooms Lake	-42.22	147.79	487	2	0	1.0	3×H43/1	2×H60		
25	Dungrove	-42.27	146.89	552	3	0	1.3	7×H21/3	2×H24/3	H30/2	
26	Butlers Gorge	-42.28	146.33	682	2	1	0.6	7×H21/3	H66		
27	Oatlands	-42.30	147.38	402	2	0	0.6	H43/1	7×H60		
28	Tin Dish Rivulet	-42.31	147.44	412	4	0	3.0	H24/3	H30/2	H43/1	2×H60
29	Osterley	-42.35	146.74	347	2	1	0.7	6×H24/3	H56		
30	Bothwell Lake Bignells	-42.38	147.00	370	2	0	0.8	8×H24/3	2×H30/2		
31	Bothwell	-42.40	147.10	481	1	0	0.0	8×H24/3			
32	Ellesmere	-42.40	147.30	422	3	1	2.0	2×H30/2	2×H59	H61	
33	Stonor	-42.43	147.43	444	4	2	1.9	H43/2	5×H62	H63	H65
34	Uralla	-42.55	146.86	193	4	2	2.5	H19/2	H21/4	H24/6	3×H30/2
35	Curringa	-42.57	146.77	100	2	0	0.6	7×H21/3	H30/2		
36	Gatehouse Marsh	-42.59	147.78	41	6	3	4.0	H19/1	H21/1	H21/2	H24/2
								H24/4	H43/2		
37	South Arm	-43.03	147.42	16	2	1	0.7	H24/2	6×H30/1		

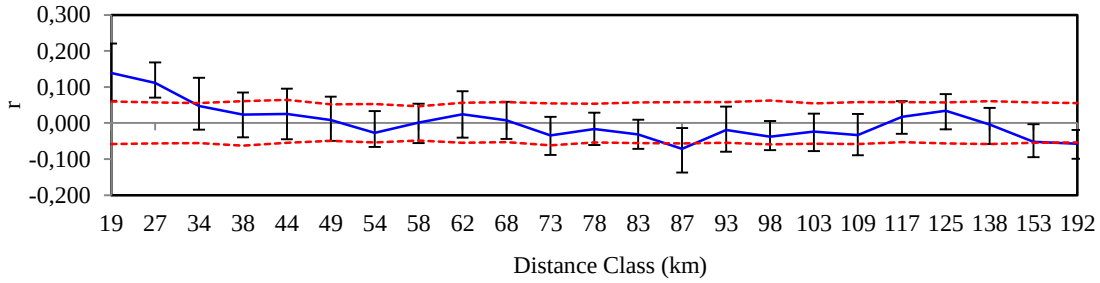
a) Results of spatial structure analysis using chloroplast markers



b) Results of spatial structure analysis based on the maternal samples using nuclear markers



c) Results of spatial structure analysis based on the progenies using nuclear markers



d) Results of fine scale spatial structure analysis based on maternal samples using nuclear markers

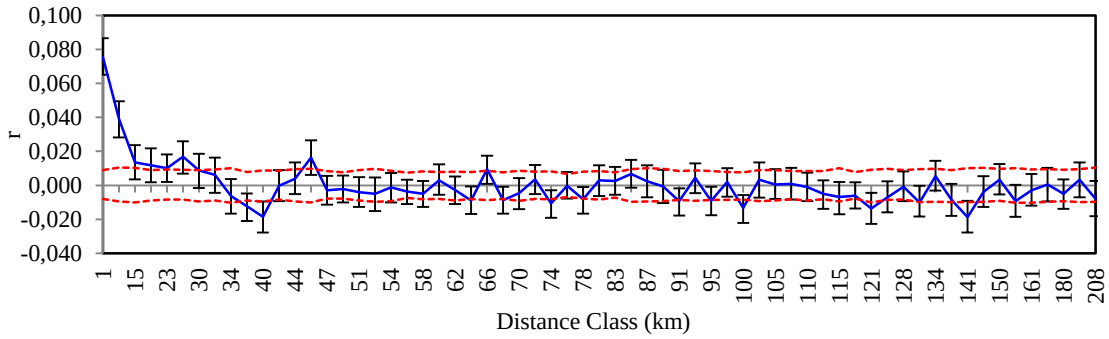


Fig. 3.2. Correlograms of *Eucalyptus pauciflora* from Tasmania based on the geographic distance and Nei's (1972) genetic distance, based on: a) the maternal populations using four polymorphic chloroplast marker; b) the maternal populations using 10 nuclear microsatellite markers; c) the progenies using 10 nuclear microsatellite markers; and d) fine scale analysis of maternal samples based on 10 nuclear microsatellite markers. *r* is the autocorrelation coefficient, upper and lower confidence limits bound the 95% confidence interval about the null hypothesis of no spatial structure for the combined data set as determined by the permutation.

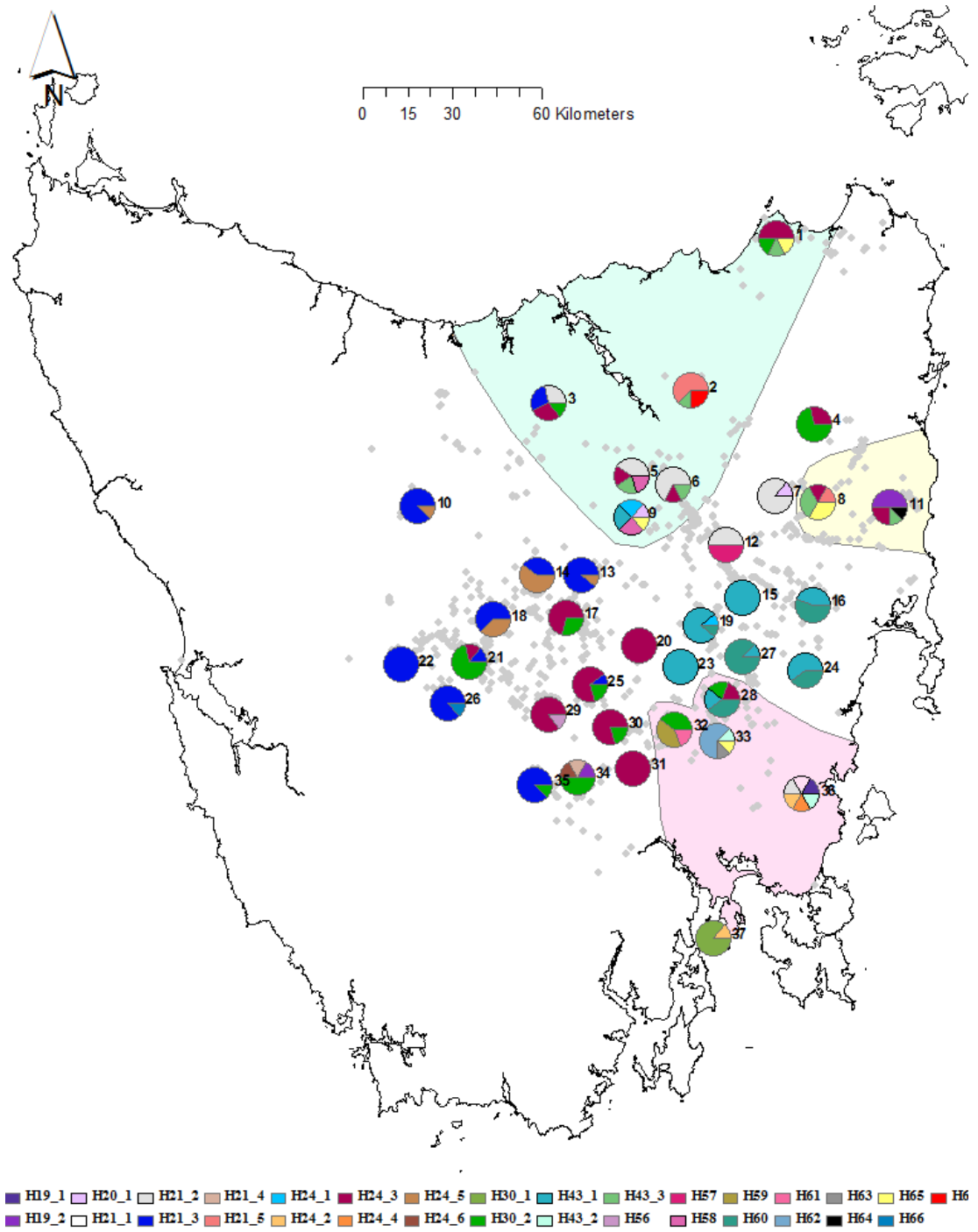


Fig. 3.3. Geographic distribution of chloroplast DNA haplotypes found in *Eucalyptus pauciflora*, superimposed on the whole species distribution (grey diamonds) in Tasmania based on the information from Williams and Potts (1996), Natural Value Atlas (www.naturalvaluesatlas.tas.gov.au) and additional records from the University of Tasmania. Population numbers correspond to those defined in Table 3.3. Pie charts represent relative proportions of each population that contains the given (colour-coded) chloroplast haplotypes. Colour code of the haplotypes corresponds to the Fig. 3.1. Note that the chloroplast haplotypes are structured geographically. Three coloured regions group populations hypothesised to have been in glacial refugia.

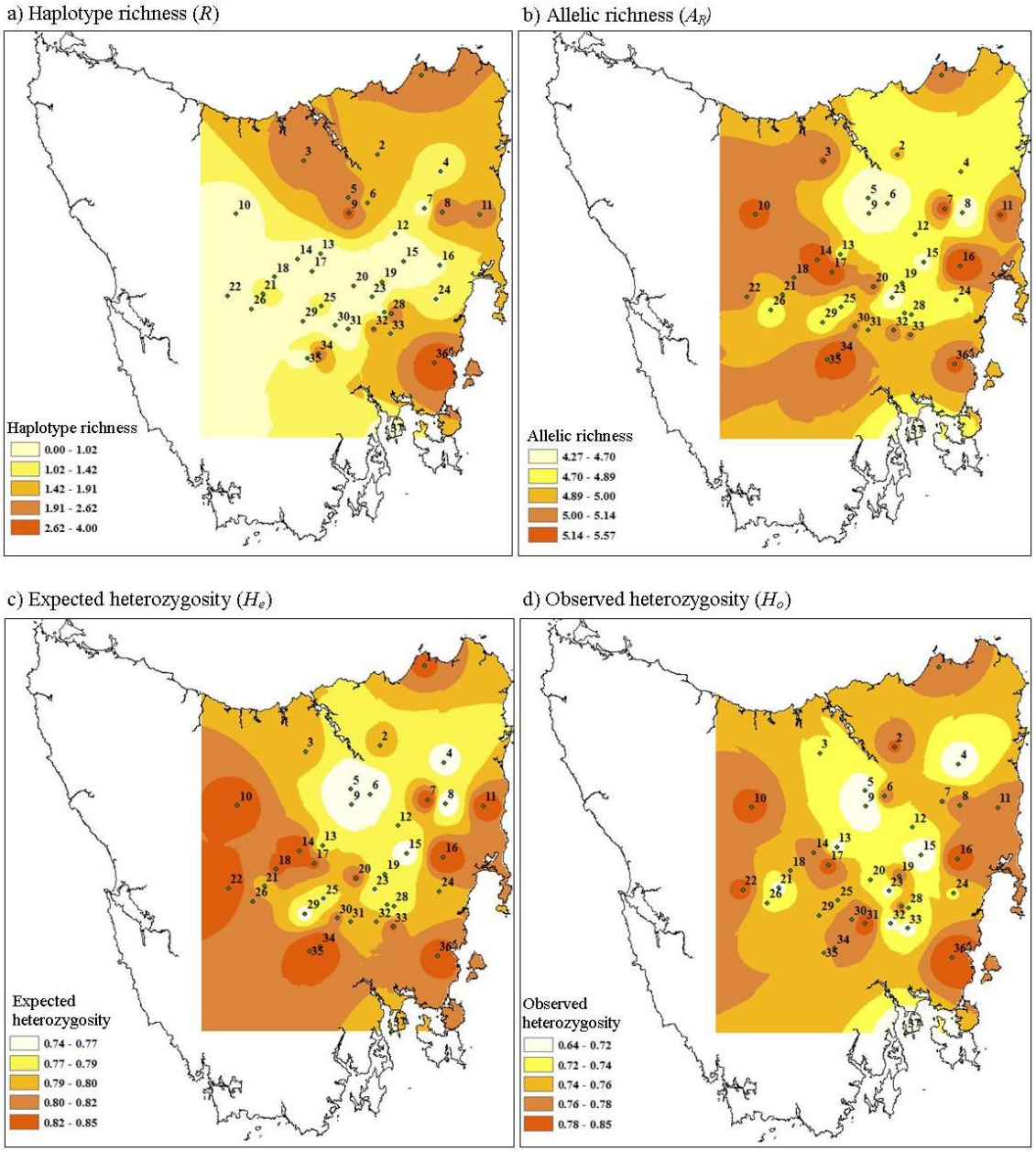


Fig. 3.4. Geographic variation in chloroplast and nuclear diversity parameters for populations of *Eucalyptus pauciflora*. The maps show spatial variation in population means for a) chloroplast haplotype richness per population (rarified) (*R* in Table 3.3); b) nuclear microsatellite allelic richness (rarefied) per population (A_R, Table 3.5); c) nuclear microsatellite expected heterozygosity (*He*, Table 3.5); and d) nuclear microsatellite observed heterozygosity (*Ho*, Table 3.5). The contour mapping is based on an inverse distance weighted interpolation (IDW) between data points. The numbers against each point are the population identification numbers (ID) given in Table 3.3. Note the figures shows many discrepancies between chloroplast and nuclear diversity.

Table 3.4. Regression analysis testing the effect of altitude on population-level genetic diversity parameters and also to test the correlation between population-level chloroplast diversity (haplotype richness) and nuclear diversity parameters (allelic richness, expected heterozygosity, observed heterozygosity and the fixation index) in Tasmanian *Eucalyptus pauciflora*.

Diversity parameter	Altitude								Correlation with haplotype richness (*R*)	
	Maternal				Progenies					
	R^2 (%)	relationship	$F_{1,35}$	*P*	R^2	relationship	$F_{1,35}$	*P*	r	*P*
Haplotype richness, *R*	0	NA	0.01	0.912	NA	NA	NA	NA	NA	NA
Allelic richness, A_R	13.2	positive	5.3	0.027	45.2	positive	28.9	0.000	0.01	0.951
Expected heterozygosity, *He*	11.8	positive	4.7	0.037	19.7	positive	8.6	0.006	-0.09	0.582
Observed heterozygosity, *Ho*	0.01	NA	0.5	0.471	11.7	positive	4.7	0.038	0.09	0.598
Fixation index, *F*	0	NA	0.3	0.566	0	NA	0.05	0.822	-0.16	0.352

3.3.2 Nuclear microsatellites

Of the 13 nuclear microsatellites screened, EMBRA08, CRC6 and ES157 had many spurious peaks, so were dropped from further analysis. Technical replicates of 5% of the samples showed an error rate of 4.6 % per allele in the final set of 10 loci analysed. The 10 loci used for the study were highly variable in the 276 maternal samples, with a total of 229 alleles scored. Each locus had from nine (ES255) to 47 (EMBRA196) alleles, with a mean of 23 alleles per locus (Table 3.5). In the 1,359 progenies sampled, a total of 270 alleles were observed, with a mean number of 27 alleles per locus, ranging from 11 (ES255) to 53 (EMBRA196) alleles. The expected heterozygosity (*He*) was higher than the observed heterozygosity (*Ho*) in all loci, both in maternal samples and in progenies. The total diversity (H_T) in maternal trees and progenies was the same (mean *He or* H_T; maternal = 0.83, progenies = 0.83), however, observed heterozygosity in progenies was less than the maternal samples (maternal *Ho* = 0.75: progenies *Ho* = 0.70). In maternal samples, F_{IS} was positive for all loci except CRC11 where F_{IS} was slightly negative (-0.01). In the progenies, none of the F_{IS} values were negative. For all loci, estimates of F_{IS}, F_{IT} and F_{ST} were higher in progenies than in the maternal samples (Table 3.5).

The population level genetic diversity statistics for maternal samples and progenies are given in Table 3.6. Expected heterozygosity averaged over loci was high for all populations for maternal samples and progenies (maternal *He* = 0.80: progenies *He* = 0.79). In virtually all maternal populations (32 out of 37) expected heterozygosity was higher than observed heterozygosity. While the overall average expected heterozygosity in progenies was essentially the same as that of maternal samples, the observed heterozygosity in progenies was slightly lower than in maternal populations (maternal *Ho* = 0.80: progenies *Ho* = 0.79). For all but four of the populations, maternal fixation indices were positive and close to zero, with an average of 0.07 indicating the presence of slightly more homozygosity than expected under random mating. Population-level fixation indices for the progenies were all positive and the average (0.12) was slightly higher than the average fixation index for maternal samples, indicating slightly greater inbreeding in the progenies. Similarly, private alleles were observed in many populations (20 out of 37; data not shown), but the distribution of private alleles did not show any geographic pattern.

AMOVA indicated that low but significant molecular genetic variation occurred among populations (maternal = 3%; progenies = 5%; $P < 0.001$). Similarly, the overall F_{ST} for the maternal populations was low (0.03±0.003), half that observed in the progenies (0.055±0.003). Using nuclear and cpDNA estimates of population differentiation, the ratio of pollen- to seed-mediated gene-flow was estimated at 24.4 for the maternal samples and 14.7 for progenies. This result indicates that pollen dispersal is the predominant contributor to gene flow in *E. pauciflora.*

A Mantel test of the correlation of pairwise F_{ST} values from maternal and progeny estimates was highly significant (n = 37; Mantel r = 0.80, $P < 0.001$), with the positive correlation indicating similar patterns of population differentiation across generations. UPGMA dendrograms constructed using Nei's (1972) genetic distance did not reflect any clear spatial grouping of populations for either maternal or progeny data (data not shown). However, the South Arm (37) and Butlers George (26) populations were outliers in both dendrograms. Similarly, a Bayesian STRUCTURE analysis (results not shown) of maternal samples did not detect any geographic clustering of the genetic diversity. With every cluster partition starting at K = 2, populations were admixed and

Table 3.5. Genetic parameters for ten nuclear microsatellite loci in Tasmanian *Eucalyptus pauciflora*. 'Source' refers to the result for either maternal (Mat) or progeny (Prog) samples; *Rppt* (%) = repeatability percentage; *A*(*0*) = frequency of null alleles; n = number of scored individuals per locus; *A* = observed number of alleles per locus; *He* = expected heterozygosity; *Ho* = observed heterozygosity; *Fit*, *Fst*, *Fis* are the total, between- and within-population inbreeding coefficients, respectively, and S.E. is their standard error. Percent repeatability estimates and null allele frequencies were calculated from combined maternal and progeny data. Note that the mean of H_e across loci is equal to H_T.

Locus	Source	*Rppt* (%)	*A*(*0*)	n	*A*	*He*	*Ho*	*Fit*	S.E	*Fst*	S.E	*Fis*	S.E
CRC11	Mat	97.3	0.005	276	25	0.91	0.89	0.02	0.02	0.02	0.01	-0.01	0.02
	Prog			1344	27	0.91	0.83	0.10	0.02	0.05	0.01	0.05	0.01
EL13	Mat	98.2	0.053	276	12	0.47	0.39	0.17	0.05	0.03	0.02	0.14	0.05
	Prog			1339	17	0.47	0.36	0.27	0.03	0.05	0.01	0.23	0.03
Embra011	Mat	93.8	0.034	273	27	0.92	0.79	0.14	0.02	0.03	0.01	0.11	0.02
	Prog			1301	29	0.92	0.71	0.24	0.02	0.06	0.01	0.20	0.02
EMBRA187	Mat	93.3	0.057	275	22	0.92	0.73	0.21	0.03	0.04	0.01	0.17	0.03
	Prog			1279	25	0.91	0.66	0.28	0.02	0.06	0.01	0.23	0.02
EMBRA196	Mat	88.8	0.031	274	47	0.93	0.81	0.14	0.03	0.04	0.01	0.10	0.03
	Prog			1276	53	0.93	0.72	0.24	0.02	0.07	0.01	0.18	0.02
EMBRA210	Mat	98.3	0.017	276	24	0.87	0.77	0.12	0.02	0.03	0.01	0.09	0.03
	Prog			1327	29	0.88	0.78	0.12	0.01	0.06	0.01	0.07	0.02
EMBRA232	Mat	93.3	0.015	275	25	0.94	0.91	0.03	0.02	0.03	0.01	0.00	0.02
	Prog			1330	31	0.94	0.84	0.11	0.02	0.06	0.01	0.06	0.02
ES140	Mat	96.4	0.015	276	22	0.77	0.74	0.03	0.03	0.02	0.01	0.01	0.03
	Prog			1290	30	0.77	0.71	0.09	0.02	0.04	0.01	0.05	0.02
ES211	Mat	96.4	0.012	276	16	0.78	0.73	0.06	0.03	0.04	0.02	0.02	0.03
	Prog			1334	18	0.80	0.70	0.13	0.02	0.07	0.01	0.07	0.02
ES255	Mat	98.2	0.06	276	9	0.80	0.72	0.11	0.03	0.05	0.01	0.06	0.03
	Prog			1300	11	0.81	0.66	0.19	0.02	0.07	0.01	0.13	0.02
Mean	Mat	95.4	0.03	276	23	0.83	0.75	0.10	0.02	0.03	0.003	0.07	0.02
	Prog			1312	27	0.83	0.70	0.16	0.02	0.06	0.003	0.12	0.02

the likelihood values were lower than that of K = 1, arguing for little spatial structuring of genetic variation. Nevertheless, the Mantel test for an association between Nei's (1972) genetic distance and geographic distance among populations showed a significant but weak correlation for both maternal (Mantel r = 0.22, P = 0.010) and progeny (Mantel r = 0.32, P = 0.003) samples. Spatial autocorrelation based on both maternal samples and progenies showed that this was due to populations within 27 km of each other being significantly more similar than would be expected from chance alone, but beyond that distance there was no evidence of spatial structure of genetic variation (Fig. 3.2b and 3.2c). Fine-scale spatial structure analysis using individual-level rather than population-level genetic distances for maternal samples, showed a similar

trend, but allowed the patch size to be narrowed further, with the major change in genetic distance occurring at approximately 10 km (Fig. 3.2d). Mantel tests also showed a weak positive correlation of Nei's (1972) genetic distance and the differences in altitude among populations (maternal samples r = 0.15, $P = 0.06$; progenies r = 0.19, $P = 0.03$).

The population-level genetic diversity parameters were highly positively correlated between maternal and progeny samples (*A*, $r^2 = 0.77$, $P < 0.001$; *He*, $r^2 = 0.87$, $P < 0.001$; A_R, $r^2 = 0.83$, $P < 0.001$), except for observed heterozygosity (*Ho*, $r^2 = 0.45$, $P = 0.004$) and the fixation index (*F*, $r^2 = 0.27$, $P = 0.136$). The populations varied markedly in nuclear genetic diversity. Western high altitude (460 m to 1138 m) populations (10, 14, 17, 20, and 22) had high allelic richness (Fig. 3.4b) and heterozygosity (Fig. 3.4c). This relatively high nuclear diversity contrasted with the low chloroplast haplotype richness in these populations (Fig. 3.4a). Populations exhibiting high haplotype and high nuclear genetic diversity, for example the northern-most coastal population (1) and two eastern populations (11 and 36), appear to be 'hot spots' of genetic diversity. The centre of high chloroplast haplotype richness in this area (5, 6 and 9) was clearly contrasted by the depauperate nuclear diversity of the same populations. While there was no association of chloroplast haplotype diversity with altitude, there was a positive relationship between altitude and nuclear microsatellite diversity (Table 3.4), particularly notable in the western central highland area (14, 17, 18, and 20). For the maternal samples, a regression analysis showed that population heterozygosity (H_e) increased with increasing altitude ($P = 0.037$), and this trend was even more apparent with the progenies ($P = 0.006$). Allelic richness similarly increased with altitude (maternal samples $P = 0.027$: progenies $P < 0.001$). The contrasting patterns of variation in chloroplast haplotype richness and nuclear diversity were reflected by there being no significant correlation between the chloroplast haplotype richness and any of the nuclear diversity parameters among populations of *E. pauciflora* (Table 3. 4).

Table 3.6. Genetic parameters for Tasmanian *Eucalyptus pauciflora* populations estimated for the maternal samples (Mat) and progenies (Prog) and averaged across 10 nuclear microsatellite loci: n = number of scored individuals per population; *A* =observed number of alleles per locus; *He* =expected heterozygosity; *Ho* =observed heterozygosity; A_R = allelic richness (standardized to a sample size of 4); *F* = Wright's fixation index.

		n		*A*		*He*		*Ho*		A_R		*F*	
ID	Population	Mat	Prog	Mat	Prog	Mat	Prog	Mat	Prog	Mat	Prog	Mat	Prog
1	Waterhouse	6	38	6.4	11.0	0.82	0.79	0.78	0.73	5.07	4.71	0.05	0.07
2	Nunamara	8	38	7.3	11.2	0.8	0.78	0.79	0.65	4.91	4.70	0.01	0.17
3	Brushy Lagoon	7	37	7.4	12.3	0.8	0.81	0.74	0.70	5.15	4.99	0.08	0.13
4	Tyne River	6	28	6.1	9.10	0.76	0.73	0.68	0.56	4.74	4.32	0.11	0.24
5	Longford	10	37	7.1	9.40	0.74	0.70	0.69	0.61	4.34	4.07	0.07	0.13
6	Symmons Plains	6	36	5.5	9.00	0.76	0.72	0.78	0.62	4.43	4.06	-0.03	0.13
7	Rossarden	7	38	7.6	14.1	0.84	0.82	0.76	0.72	5.29	5.08	0.10	0.12
8	Avoca	6	37	5.2	9.20	0.74	0.75	0.77	0.72	4.27	4.27	-0.03	0.03
9	Cressy	8	38	6.8	10.2	0.74	0.73	0.66	0.66	4.59	4.38	0.11	0.09
10	Lake Rowallan	8	38	8.0	12.7	0.83	0.82	0.80	0.75	5.19	4.97	0.04	0.08
11	Dukes Marshes	8	36	7.8	12.6	0.83	0.82	0.78	0.68	5.17	5.04	0.07	0.17
12	Conara	8	37	7.2	11.5	0.79	0.76	0.74	0.70	4.90	4.62	0.06	0.08
13	Lake Arthur	9	35	7.7	12.1	0.78	0.77	0.70	0.68	4.83	4.74	0.11	0.12
14	Great Lake	10	37	9.0	13.8	0.84	0.84	0.78	0.74	5.35	5.30	0.07	0.12
15	Ross	10	35	7.5	11.4	0.75	0.78	0.64	0.69	4.60	4.68	0.16	0.11
16	Lake Leake	9	38	9.7	14.4	0.85	0.84	0.82	0.76	5.57	5.26	0.03	0.09
17	Wihareja	7	39	7.8	12.7	0.82	0.83	0.81	0.74	5.43	5.22	0.01	0.11
18	Pine Tier	8	35	7.6	11.7	0.82	0.79	0.75	0.68	5.06	4.75	0.09	0.13
19	Tunbridge	9	38	7.4	10.4	0.78	0.75	0.78	0.68	4.79	4.46	0.0	0.09
20	Interlaken	8	36	7.8	13.7	0.82	0.82	0.73	0.73	5.17	5.13	0.12	0.11
21	The Point	7	36	6.9	12.8	0.79	0.78	0.70	0.69	4.93	4.88	0.12	0.12
22	Lake St Clair	9	35	7.6	12.1	0.84	0.82	0.8	0.78	5.05	5.02	0.05	0.05
23	Woodbury Hill	5	28	5.0	9.50	0.77	0.75	0.70	0.69	4.41	4.43	0.10	0.08
24	Tooms Lake	5	38	5.5	10.3	0.80	0.79	0.74	0.66	4.82	4.59	0.09	0.17
25	Dungrove	10	36	7.7	10.3	0.77	0.77	0.75	0.63	4.72	4.63	0.03	0.19
26	Butlers Gorge	8	37	6.8	12.8	0.81	0.84	0.74	0.73	4.75	5.06	0.1	0.13
27	Oatlands	8	38	6.9	11.0	0.78	0.77	0.78	0.68	4.70	4.51	0.01	0.11
28	Tin Dish Rivulet	6	38	6.2	11.4	0.77	0.76	0.73	0.69	4.80	4.56	0.06	0.10
29	Osterley	4	20	4.7	9.0	0.76	0.76	0.75	0.69	4.70	4.47	0.02	0.09
30	Bothwell Lake	10	35	8.6	11.4	0.81	0.79	0.77	0.76	5.04	4.77	0.06	0.03
31	Bignells Bothwell	7	33	7.0	10.4	0.79	0.80	0.83	0.76	4.93	4.78	-0.05	0.05
32	Ellesmere	5	36	5.9	10.5	0.8	0.78	0.7	0.66	5.10	4.71	0.14	0.15
33	Stonor	8	35	7.4	12.4	0.82	0.81	0.7	0.72	5.03	4.97	0.16	0.11
34	Uralla	5	28	6.4	11.0	0.85	0.83	0.78	0.76	5.48	5.04	0.10	0.09
35	Curringa	8	37	7.6	11.1	0.83	0.81	0.74	0.69	5.14	4.84	0.12	0.15
36	Gatehouse Marsh	6	35	6.7	9.9	0.83	0.79	0.85	0.66	5.18	4.60	-0.03	0.16
37	South Arm	7	38	5.3	9.6	0.79	0.80	0.67	0.67	4.45	4.58	0.07	0.16
	Mean	7	35	7	11.3	0.80	0.79	0.75	0.70	5.33	4.73	0.07	0.12

3.4 Discussion

The overall pattern of nuclear genetic diversity in Tasmanian *E. pauciflora* was characterized by high total diversity (H_T = 0.83), low inbreeding (F_{IS} = 0.07) and low population differentiation (F_{ST} = 0.03) in the mature forest. The level of total genetic diversity in the species was comparable to the average reported for predominantly outcrossing tree species (H_T = 0.82, Petit *et al.* 2005) and other widely distributed *Eucalyptus* species (*E. regnans* H_T = 0.82, Nevill 2010; *E. obliqua* H_T = 0.83, Bloomfield *et al.* 2011a; *E. globulus* H_T = 0.87, Jones *et al.* 2002). The high genetic diversity of trees such as eucalypts is believed to be due to efficient gene flow mechanisms and the predominance of outcrossing, which are life history traits that promote the maintenance of high diversity (Austerlitz *et al.* 2000). The F_{ST} in the Tasmanian *E. pauciflora* (0.03) was lower than the average reported for predominantly outcrossing tree species (F_{ST} = 0.14; Petit *et al.* 2005) and the average reported for many eucalypt species using nuclear microsatellite markers (F_{ST} = 0.147; Byrne 2008b). However, *E. pauciflora* populations showed slightly more genetic differentiation than the co-occurring wide-spread *E. obliqua* which was sampled similarly across its distributional range in Tasmania (F_{ST} = 0.02; Bloomfield *et al.* 2011a).

Pollen appears to be the predominant vehicle for gene flow in *E. pauciflora*, given the positive pollen-to-seed F_{ST} ratio. Seeds are dispersed mainly by gravity and/or wind in *Eucalyptus*, so that they generally fall within twice the canopy height of the maternal tree (Potts and Wiltshire 1997). While there is little published literature on the pollination biology of *E. pauciflora*, other small-flowered eucalypt species tend to be pollinated by insects and, less frequently, by birds and mammals (House 1997). The pollen- to seed-flow ratios measured in *E. pauciflora* are higher than that reported for the insect-pollinated *E. nitens* (pollen: seed F_{ST} ratio of 7.2: Byrne *et al.* 1998), are comparable with an average of 17 reported for 93 plant species (Petit *et al.* 2005), but are markedly lower than the average reported for eight other eucalypt species (145.7; Byrne 2008b; Bloomfield *et al.* 2011a).

While similar levels of total genetic diversity were observed in progenies and maternal samples, there was a discrepancy in F_{ST} between the two (maternal F_{ST} = 0.03; progenies F_{ST} = 0.06). This difference in the F_{ST} might be related to inbreeding, as seen in the

increase in inbreeding coefficient (F_{IS}) for progenies. Both of these differences could be explained by the combined presence of selfing (t_s = 0.10) and biparental inbreeding (t_m-t_s = 0.16) in the *E. pauciflora* populations (Gauli *et al.* 2014), coupled with selection against the products of inbreeding (e.g., deleterious mutations in selfed and homozygote seedlings; Patterson *et al.* 2000) between the seedling and the mature cohorts, a common phenomenon in trees (Hufford and Hamrick 2003; Naito *et al.* 2005). This hypothesis is supported further by the difference in the ratio of pollen- to seed-mediated gene flow between the mature generation and the progenies (maternal samples: 24.5, progenies: 14.7). As a forest matures, selection may act against the products of selfing and biparental inbreeding that arise from near-neighbour matings, thereby favouring the products of long distance pollen dispersal. Such selection may explain the lack of geographic structuring of nuclear genetic diversity observed in *E. pauciflora*.

The correlation between population differentiation and geographic distance between populations of *E. pauciflora* was weak. Trees within 10 km were genetically more similar to each other than would be expected from chance alone. Within a distance of 27 km, there was still an above-average nuclear genetic similarity between populations, which indicates a distance over which broad-scale pollen dispersal is likely to define the local population. Beyond this distance the degree of differentiation of populations was unrelated to their separation distance. Similarly significant but weak genetic structuring has been observed in other widespread eucalypts over quite large distances, for example, 40 km in *E. globulus* (Yeoh et al. 2012) and 51 km in *E. obliqua* (Bloomfield *et al.* 2011a). The present study has shown that pollen has a much greater dispersal capacity than seed in *Eucalyptus*, and this enables long distance gene flow between populations and, perhaps, homogenisation of nuclear genetic variation across populations, thus masking or erasing the differentiation that may have accumulated in glacial refugia. The same trend of weak but significant isolation by distance was found for chloroplast DNA. In this case, populations within 38 km were more similar in their chloroplast microsatellite affinities than average. The discrepancy between the population structures found using nuclear and chloroplast markers may reflect differences in mutation rates between nuclear and chloroplast DNA, or the fact that the chloroplast DNA maintains a stronger historical signal.

Several studies have revealed that there is a trend towards high chloroplast diversity and/or high frequency of private haplotypes in glacial refugia relative to recently colonized areas (Heuertz *et al.* 2004; Hewitt 1996; Newton *et al.* 1999). Based on this premise, our evidence suggests three glacial refugia in Tasmania: Tamar Valley (lowland populations extending from the northern midlands to the north coast); St Pauls River Valley (eastern populations in the upper Avoca valley including Dukes Marsh); and Storm Bay region (the south eastern lowland populations). Using the current climatic envelope of the species, Williams (1991) suggested that *E. pauciflora* was once widespread throughout the lowlands of south eastern Tasmania (i.e., Storm Bay region in this study). Williams (1991) further argued that *E. pauciflora* may also have had populations in the north and in the east, in regions that coincide well with areas of high haplotype diversity in this study. Further support for *E. pauciflora* glacial refugia in these areas comes from a glacial climate modelling study by Kirkpatrick and Fowler (1998) that proposed the existence of multiple small, scattered refugia in northern and south eastern Tasmania for frost resistant eucalypts such as *E. pauciflora*. Morphological variation (Williams and Ladiges 1985) and pollen studies (Dodson 1977) also suggested the present low altitude population of *E. pauciflora* are relicts from the most recent glacial period. The hypothesised glacial refugia in Storm Bay (south eastern Tamania) and the eastern region of Tasmania are consistent with the hypothesised locations of glacial refugia for many other eucalypts species (Freeman *et al.* 2001; McKinnon *et al.* 2004a; McKinnon *et al.* 2001b; Nevill *et al.* 2010; Potts and Reid 1985). In addition to the high level of chloroplast haplotype diversity in *E. pauciflora*, the St Pauls River Valley region also harbours high levels of endemism in other plant taxa, an observation consistent with an area of glacial refuge (Kirkpatrick and Brown 1984).

Low chloroplast haplotype diversity in the midlands of Tasmania is consistent with palynological study which proposed that this region was deforested during glacial periods (Sigleo and Colhoun 1981). This suggests probable migration of haplotypes from the hypothesized glacial refugia into the midlands following deglaciation. Near fixation of three different chloroplast haplotypes in the relatively high altitude populations in the central west, central and the central east regions suggests that these populations were the result of such post-glacial upslope colonization (Dodson 1977;

Williams and Ladiges 1985). Similar scenarios of post-glacial migration have been proposed in numerous other species in Europe (Demesure *et al.* 1996; Ferris *et al.* 1998; Marchelli *et al.* 1998; Petit *et al.* 1997). However, as Hope and Kirkpatrick (1989) proposed, there is a possibility that highland populations may have resulted from populations expansion out of high altitude refugia where *E. pauciflora* might have been able to survive in savannah habitats during the glaciations. Williams (1991), using climate modelling, found support for this hypothesis, because the present distribution of *E. pauciflora* occupies most of the predicted potential range which may not have been expected with post-glacial migration from lowland refugia. However, the lower chloroplast DNA diversity in broad areas of haplotype sharing amongst populations found in the central Midlands (which would have had a glacial arid environment supporting mainly grassland; Kirkpatrick and Fowler (1998)) and the high altitude Central Plateau region argues against the Hope and Kirkpatrick (1989) hypothesis of multiple small refugial populations in these areas. This study is, to our knowledge, the first to provide empirical evidence on this issue.

The combination of high nuclear microsatellite diversity combined with low chloroplast haplotype diversity in the newly colonized areas, suggests that, following de-glaciation, highland areas were colonised relatively rarely by seed, but once established these populations were able to exchange genetic material with other populations through pollen. Similar discrepancies between chloroplast and nuclear differentiation were also observed in oak species (Finkeldey and Mátyás 2003; Kremer *et al.* 2002; Petit *et al.* 2002a; Petit *et al.* 2002b) and extensive pollen flow was suggested to mask the chloroplast differentiation. Another explanation for populations having high nuclear diversity and low cpDNA diversity includes the impact of hybridisation and introgression. *Eucalyptus pauciflora* is known to hybridize with the 'ash' eucalypts, *E. obliqua*, *E. delegatensis* and *E. regnans*, as well as endemic 'peppermint' eucalypt species, including the tree-line species, *E. coccifera* (Duncan 1989; Pryor 1951; Pryor 1953; Williams and Ladiges 1985). Genetic introgression arising from such hybridisation might be expected to increase both nuclear and chloroplast genetic diversity in populations of these species. However, although they share many common haplotypes, suggesting common ancestry, the absence of common geographic patterns of haplotype diversity suggests the absence of introgression, at least with the ash species.

However, the possibility of hybridisation and introgression with other co-occurring peppermint species which have-not been studied cannot be dismissed.

3.5 Conclusion

This study has shown that Tasmanian populations of *E. pauciflora* have high levels of genetic diversity of both chloroplast and nuclear loci. There was little population differentiation in nuclear markers, but there was above-average genetic similarity in populations within 27 km, which indicates a distance over which broad-scale pollen dispersal is likely to define the local population. The distribution of chloroplast haplotype diversity provides evidence of three major glacial refugia for *E. pauciflora* in the north and east of Tasmania. Haplotype affinities provided evidence of migration of populations from north and east towards the south and west of Tasmania and from lowland areas to highland areas. Higher nuclear diversity in newly colonised areas compared to lowland refugial regions, and the contrary in chloroplast DNA markers, suggest bottlenecks in seed dispersal in newly colonised regions, but persistent high gene flow between immigration zones through pollen dispersal. Our results provide evidence against the hypothesis that central highland populations of *E. pauciflora* originate from *in situ* high altitude refugia.

3.6 Acknowledgements

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Chapter 4. Evidence for climate adaptation in early-life cycle traits of a wide-spread eucalypt

Abstract

Understanding the genetic basis of adaptation to contemporary environments is fundamental to predicting the evolutionary responses of tree species to future climates and thus is important in providing guidance for forest restoration and translocation activities. We studied the adaptive potential of the widespread *Eucalyptus pauciflora*, a tree species of increasing interest for restoration purposes due to its capacity to withstand harsh environments. Using seedlings grown in a glasshouse, we assessed quantitative genetic variation of seedling traits in 275 open-pollinated families collected from 37 populations native to the island of Tasmania and studied the association of this variation with climatic factors. Most traits exhibited significant genetic variation both within and between populations. Significant spatial genetic structure was detected within 27 km, suggesting an operational limit for the definition of a 'local' population. While there was no association of genetic distance with geographic distance for populations separated by greater spatial distances, there were significant associations with altitudinal and climatic differences over the full range of values. Strong evidence of adaptation to local environments was found for traits associated with ontogenetic maturation and resource allocation, as well as stem oil glands and leaf colour. This evidence was high quantitative inbreeding coefficients (Q_{ST}) estimates and strong correlations with climatic factors, especially for maximum temperature of the warmest month and moisture indices. Populations originating from hotter, drier regions exhibited greater lignotuber development, delayed ontogenetic development, reduced oil gland development and had lighter green leaves than populations from cooler, wetter regions. While many traits exhibited parallel responses to the same climatic variables, analysis of intra- and inter-population genetic correlations indicated that these were likely to be independent responses, controlled by different genes, rather than correlated responses to selection arising from pleiotropy or linkage. It is argued that small changes in climate, such as a 1ºC change in the maximum temperature of the warmest month, are likely to lead to mal-adaptation of local populations of the species. However, additive variation and heritability within populations was maintained for many key adaptive traits, arguing

that populations maintain significant evolutionary potential from the standing genetic variation.

Keyword: *Eucalyptus pauciflora*, seedling traits, climatic variables, genetic correlation, adaptation

4.1 Introduction

The response of plants to climate change are being discussed widely (Aitken *et al.* 2008; Duputié *et al.* 2012; Hoffmann and Sgrò 2011; McKenney *et al.* 2007; 2007; Sgrò *et al.* 2011). The three possible alternatives are a shift of the plant to a better habitat following the changing environment, persistence through a plastic response and/or adaptation to the altered habitat, or population extinction (Aitken *et al.* 2008; Parmesan 2006; Petit *et al.* 2008). Persistence of the tree species through glacial and post glacial periods during the Pleistocene and the Holocene (Davis and Shaw 2001; Hofreiter and Stewart 2009; Petit *et al.* 2008), as well as studies of plant migration (Lloyd 2005; Mimura and Aitken 2007; Villarreal *et al.* 2012), have provided evidence of the capacity of these species to cope with climate change through a combination of adaptation (involving a genetic change), phenotypic plasticity and geographic range shift. However, with rapidly changing environment, tracking favourable habitat through migration or adaptation might not be easy, particularly for tree species because of their longevity, sedentary nature (Kremer *et al.* 2012) and the complex biotic interactions involved (Bailey *et al.* 2014). Thus prediction of the potential of a species to genetically adapt to a changed environment requires the integration of knowledge from individual level genetic to ecosystems level interactions (Bailey *et al.* 2014).

The ability of species to respond to changing climate is likely to vary widely as a consequence of variation within and among species in their degree of phenotypic plasticity and their potential for genetic adaptation, the later in turn depending on the amount of standing genetic variation, the adaptive potential of this variation and the ability to redistribute genetic diversity through recombination and probably inter-population gene flow (Hancock *et al.* 2011). The response of a population to selection cannot be predicted on the basis of the additive genetic variance of single traits alone (Guillaume 2011). Instead, several factors need to be considered in order to predict the

adaptive potential of a species: the extent to which traits are genetically correlated; the additive genetic variance among individuals in each trait; and the direction and intensity of selection (Hellmann and Pineda-Krch 2007). Although the presence of genetic correlations among traits is often regarded as a constraint to adaptation (Etterson and Shaw 2001), their effect depends on the direction of selection and the multidimensional pattern of trait variation (Guillaume 2011), and genetic correlations can also facilitate adaptation (Agrawal and Stinchcombe 2009). Thus both multivariate trait space and univariate responses need to be well understood before the adaptive response of a species can be predicted. However, for most tree species, empirical data on patterns of genetic correlations among key ecological traits and on the spatial and temporal variation of their joint selection pressures is missing (Kremer *et al.* 2012).

Eucalypts are foundation species of many forest and woodland ecosystems across the Australian continent (Williams and Woinarski 1997). Eucalypts are well-known for the large amount of genetic diversity which may occur within species in their native range (Potts and Wiltshire 1997). This genetic variation may be geographically dispersed at a broad-scale (e.g. population or racial- Dutkowski and Potts 1999), as local differentiation over steep environmental gradients (Foster *et al.* 2007) or as fine-scale genetic differentiation over just metres (Jones *et al.* 2007).

Eucalyptus pauciflora, the iconic 'snow gum' of mainland Australia, has the widest altitudinal range of any eucalypt species, forming the tree-line on most of the mountains of the Australian Alps and also extending almost to sea level (Boland *et al.* 2002; Williams 1991; Williams and Potts 1996). Owing to its ability to withstand very cold temperatures, dry winds and periodic drought, and given these stresses, *E. pauciflora* performs remarkably well in restoration field trials (Close *et al.* 2010). It has been selected as a key species for ecological restoration in the drought prone midlands of Tasmania, where intensive farming over 200 years has resulted in severe land degradation and tree decline (Bailey *et al.* 2013). While current guidelines favour the use of local seed sources for such restoration (Broadhurst *et al.* 2008; Mortlock 2000), local seed may no-longer be the best due to multiple factors, including forest fragmentation resulting in inbreeding in the open-pollinated seed (see Chapter 2), direct anthropogenic modification of the environment (e.g. soils), new pests and diseases, as

well as global climate change (Jones 2013; Lesica and Allendorf 1999). Global climate change is receiving increasing attention in restoration research as a key consideration in the choice of seed source (Hancock and Hughes 2012; Harris *et al.* 2006; McKay *et al.* 2005). In the present case, an indication of its importance can be gauged from understanding the extent to which climate variation across the range of *E. pauciflora* has structured the genetic variation in functional traits, and whether local populations retain genetic variation to adapt to climate change.

The present chapter explores the climate adaptation and adaptive potential of *E. pauciflora* through assessing the pattern of quantitative genetic variation in 25 seedling traits using a glasshouse trial established with seeds collected across the natural distribution of the species in Tasmania. The chapter studies the spatial patterns of genetic differentiation between populations, and whether this differentiation is adaptive and associated with altitude and climate variation across the species range. The levels of additive genetic variation within populations is assessed and the genetic covariance amongst traits is studied to determine whether parallel patterns of trait variation across the range of the species are likely caused by pleiotropy/linkage or correlated response to selection.

4.2 Materials and methods

4.2.1 Sampling sites and experimental design

Open-pollinated seeds were collected from 5 to 10 trees from each of 37 populations from Tasmania giving a total of 275 families, covering the full geographic and altitudinal range of the species on the island (Table 4.1, Fig. 4.1). The minimum distance between trees was approximately 100 m. During sample collection, the altitude, latitude and longitude of each tree were recorded. This information was used to derive estimates of climatic parameters for each tree using ANUCLIM version 6.1 software (Xu and Hutchinson 2010) which were averaged to provide population level values**.** These seedlots were grown in a glasshouse trial which also included 14 bulk open-pollinated seedlots of *E. pauciflora* from mainland Australia and 60 open-pollinated families of a Tasmanian co-occurring species, *E. tenuiramis*. Seedlings were pricked and raised in the nursery, when the plants had expanded two nodes. The seedlings were then grown in a glasshouse trial comprising three replicates and nine incomplete blocks

(i.e. trays), with each family represented once per replicate and randomised into the incomplete block design using CycDesign 4.0 (Whitaker *et al.* 2002). Altogether 432 plants were assigned in each replication, but only the 279 open-pollinated *E. pauciflora* families from Tasmania are used in the present study. In total 1,296 seedlings were measured during this experiment.

Table 4.1. Tasmanian populations of *Eucalyptus pauciflora* used for the study. Numerical codes, latitude, longitude, altitude (metres) and number of parent trees sampled from each population are shown.

Code	Population	Latitude	Longitude	Altitude (m)	Number of trees sampled
1	Waterhouse	-40.9098	147.6599	16	6
2	Nunamara	-41.3728	147.3215	405	6
3	Brushy Lagoon	-41.4094	146.7470	282	7
4	Tyne River	-41.4709	147.8171	297	7
5	Longford	-41.6302	147.0973	159	8
6	Symmons Plains	-41.6594	147.2491	166	5
7	Rossarden	-41.6888	147.6950	731	7
8	Avoca	-41.7095	147.8345	237	6
9	Cressy	-41.7193	147.1046	160	8
10	Lake Rowallan	-41.7218	146.2185	460	7
11	Dukes Marshes	-41.7225	148.1279	498	8
12	Conara	-41.8416	147.4635	206	8
13	Lake Arthur	-41.9565	146.8769	1004	9
14	Great Lake	-41.9868	146.6991	1138	10
15	Ross	-42.0017	147.5323	240	10
16	Lake Leake	-42.0211	147.8173	597	9
17	Wihareja	-42.0614	146.8143	895	6
18	Pine Tier	-42.0937	146.5166	818	8
19	Tunbridge	-42.1249	147.3646	229	9
20	Interlaken	-42.1461	147.1412	818	8
21	The Point	-42.1929	146.4222	674	7
22	Lake St Clair	-42.2014	146.1422	816	9
23	Woodbury Hill	-42.2124	147.2828	626	6
24	Tooms Lake	-42.2205	147.7928	487	5
25	Dungrove	-42.2664	146.8861	552	9
26	Butlers Gorge	-42.2792	146.3304	682	8
27	Oatlands	-42.3013	147.3842	402	8
28	Tin Dish Rivulet	-42.3079	147.4370	412	6
29	Osterley	-42.3543	146.7408	347	8
30	Bothwell Lake	-42.3798	146.9954	370	10
31	Ellesmere	-42.4014	147.2977	422	5
32	Bignells Bothwell	-42.4014	147.0962	481	8
33	Stonor	-42.4277	147.4316	444	8
34	Uralla	-42.5462	146.8591	193	6
35	Curringa	-42.5698	146.7721	100	8
36	Gatehouse Marsh	-42.5949	147.7810	41	6
37	South Arm	-43.0341	147.4223	16	6

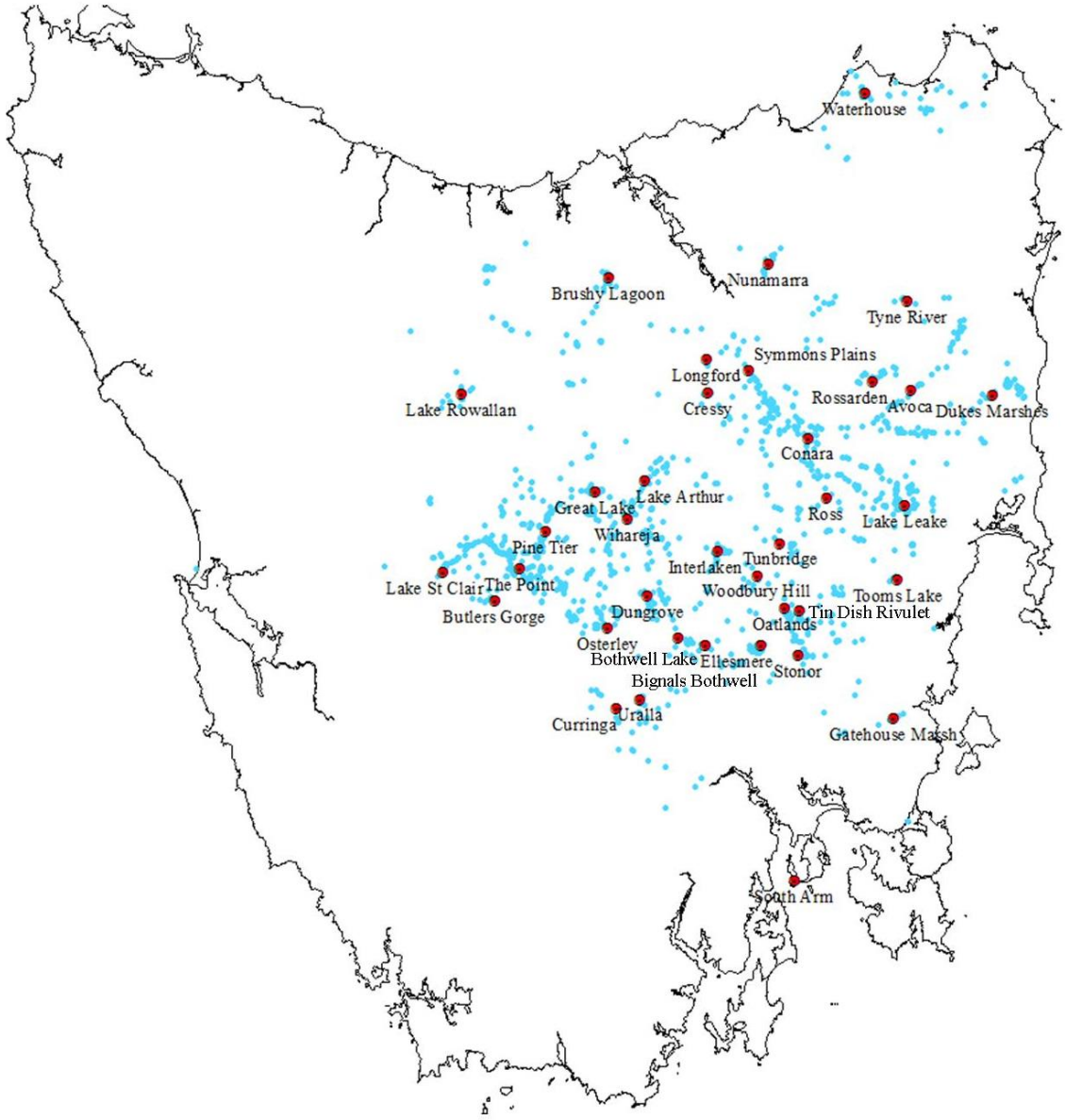


Fig. 4.1. Distribution and seed collection sites of *E. pauciflora* in Tasmania. Information on Tasmanian distribution of *Eucalyptus pauciflora* is based on the Williams and Potts (1996), Natural Value Atlas (www.naturalvaluesatlas.tas.gov.au) and additional records from the University of Tasmania. Population details are given in Table 4.1.

4.2.2 Traits measured

A series of morphological traits assessing growth (height, nodes expanded, stem diameter), facets of whole plant development (lignotuber size, leaf ontogeny), and seedling leaf morphology were assessed at 2 and 6 months. Details of measured traits are given in Table 4.2. Many of the traits assessed, were recorded previously by other authors (Turner et al. 2001; Williams and Ladiges 1985; Wiltshire et al. 1992). To be consistent with the previous studies in E. *pauciflora* (Williams and Ladiges 1985) the cotyledonary (cot) node was identified as 1. Most *Eucalyptus* species including *E. pauciflora* are heteroblastic; they have a discrete vegetative phase change between seedling and adult forms (Euclid 2006). Leaves in adult eucalypts are completely different in shape size and other characteristics. This makes eucalypts especially suitable for genetic study of the timing of developmental events (Jordan *et al.* 1999). Traits like development of petiolate from sessile leaves as well as changes from opposite to alternate leaves were included in the study to examine the marked ontogenetic change in this species. In addition, the adult leaf of *E. pauciflora* has parallel venation while juvenile leaves have venation with acute angles, so the transition between these was also recorded. A leaf at the fourth node was removed at the time of first scoring and a leaf at the eleventh node was removed at the time of second scoring and pressed onto a labelled sheet of paper for later measurement of leaf traits. The leaves were later photocopied and leaf dimensions measured using object J and image J (Abramoff *et al.* 2004).

4.2.3 NIR spectroscopy

Following O'Reilly-Wapstra *et al.* (2013), near infra-red (NIR) spectroscopy was used to obtain a holistic measure of the foliar physio-chemical differences between populations (see also Foley *et al.* 1998). Leaves at the eleventh node (or nearest node when the 11^{th} was damaged) were collected from one seedling per family (all from one replicate) for near infrared reflectance spectroscopy (NIRS). Collected leaves were freeze dried and then their surface was scanned using NIRS. A Bruker MPA Fourier Transform near infrared reflectance spectrometer coupled with a fibre-optic probe was used for scanning and Opus 5.5 (OPUS LAB) used to score the NIR spectra. Spectra were collected between 9000-4000 cm^{-1} at a resolution of 4 cm^{-1}. Two different areas on the adaxial surface of each leaf were scanned, four spectral measurements were taken at

each area, and the average was taken as a single spectrum for a leaf. Four samples were removed because they were outliers.

4.2.4 Morphological data analysis

4.2.4.1 Analysis of genetic variation between populations

Trait measurements were transformed where necessary to optimise the normality and homogeneity of variances. For each trait, the following mixed model was fitted with population as a fixed effect for all analysis:

$$Y = \text{rep} + \textit{tray(rep)} + \textit{col(tray)} + \text{population} + \textit{family(population)} + \textit{residual} \ldots \text{model (I)}$$

Where, Y is an observation of the seedling trait, rep is replicate as a fixed effect, *tray(rep)* represents the random tray within replicate effect, *col(tray)* represents the random column within tray effect, population is the fixed population effect and *family(population)* is the random family within population effect. The random *family(population)* variation was used as the error to test the fixed population effect using a Walds F-test. The significance of the *family (population)* variance component from zero was tested using a Z-test. This model was fitted for univariate analyses using PROC MIXED in SAS 9.2 (SAS Institute Inc. 2009) and the population least-square means estimated. To test for a relationship of the population least-squares means for each trait with population altitude as well as the 35 ANUCLIM climatic variables, a univariate analysis of covariance was undertaken using PROC GLM of SAS. To account for multiple testing of climate associations, the regression probabilities for each trait were adjusted for a dependent false discovery rate using PROC MULTITEST in SAS 9.2 (SAS Institute Inc. 2009).

To obtain an overall estimate of the differentiation between populations, discriminant function analysis was undertaken using the 25 seedling traits listed in Table 4.2. This analysis was undertaken using PROC DISCRIM of SAS with the pooled within-population covariance matrix and population positions on the two major discriminant axes (CV1 and CV2) plotted. The Mahalanobis generalized distances amongst populations in this discriminatory space was also calculated.

Table 4.2. Seedling traits scored on each seedling of *Eucalyptus pauciflora* for quantitative genetic analysis. The table contains the description of the traits measured, codes used for each trait in the subsequent tables and text, and transformation used during analysis.

Description	Code	Transformation
Growth and developmental traits		
Height at 2 months (cm)	HT1	
Height at 6 months (cm)	HT2	
No. of nodes with lignotubers at 2 months	LIGNO1	
No. of nodes with lignotubers at 6 months	LIGNO2	
No. of nodes at 2 months (cotyledon = 1)	NNODES1	
No. of nodes at 6 months (cotyledon = 1)	NNODES2	
Node at which 1st petiolate leaf occurs at 6 months	PET2	(NNODES2 - PET2)/NNODES2
Node at which 1st leaf alternates at 6 months	ALT2	(NNODES2 - ALT2) /NNODES2
Node at which 1st leaf twists at 6 months	TWIST2	(NNODES2 - TWIST2)/NNODES2
Other traits		
Stem oil glands (0 = absence of oil glands; 1 = presence of small oil glands 2 = protruding oil gland density)	Stem OG	
Stem diameter at node 1 (x) (cm) at 6 months	STMD	
Lignotuber diameter at node 1 (90° to x) (cm)	Lig size	√ (LIGD- STMD)/STMD
Leaf traits scored at 4th node leaf		
Leaf colour at node 4 at 2 months (1 = light green, 2 = intermediate, 3 = dark green)	Leaf colour	
Leaf lamina length (cm)	LL4	
Lamina width (cm)	LW LL4	$\log_{10}$(LW4/LL4)
Leaf area (cm^2)	LA4	
Lamina length to widest point from base (cm)	LWP LL4	LWP4/LL4
Petiole length (cm)	PL4	$\log_{10}$ (PL4)
Vein angle (°)	VA4	
Leaf traits scored at 11th node leaf		
Leaf lamina length (cm)	LL11	
Lamina width (cm)	LW LL11	$\log_{10}$(LW11/LL11)
Leaf area (cm^2)	LA 11	
Lamina length to widest point (cm)	LWP LL11	LWP 11/LL11
Petiole length (cm)	PL11	$\log_{10}$ (PL11)
Vein angle (°)	VA11	

The association of overall population differentiation with geography, altitude and climatic variables was assessed in two ways. Firstly, the independent variables were fitted as vectors into the space defined by the significant discriminant axes. This vector fitting was undertaken using the envfit function in the vegan package in R (Team 2010), which provided estimates of the direction of variation in the discriminant space, the correlation and tests of significance was calculated using 9999 permutations. Secondly, the association between the population Mahalanobis generalised distance matrix and Euclidean distance matrices representing population differences in geographic distance, altitude and climatic variables was determined using the Mantel test and autocorrelation

analysis. The overall differences in climatic variables amongst populations were calculated after standardising each variable to a mean of zero and standard deviation of one. Mantel tests (Mantel 1967) were performed in order to test for significant correlations between the various matrices and the autocorrelation analyses used to determine the pattern of change. These analyses were performed using GENALEX 6.501 (Peakall and Smouse 2006).

4.2.4.2 Estimation of genetic parameters

Genetic parameters were estimated with a restricted maximum likelihood (REML) model using average information REML algorithm (Gilmour *et al.* 1995) implemented using ASReml 3.0 (Gilmour *et al.* 2009). For univariate analyses, an individual tree mixed model was fitted:

$$Y = \text{rep} + tray(rep) + col(tray) + row(tray) + population + tree + residual \ldots \text{model (II)}$$

where, Y is an observation of the seedling trait, rep is the fixed replicate effect, *tray(rep)* represents the random tray within replicate effect, *col(tray)* represents the random column within tray effect, *row(tray)* represents the random row within tray effect, *population* is the random population effect and *tree* is the random additive genetic effect for each seedling and *residual* is the random residual variation.

The *tree* term was defined using a pedigree file to calculate the additive relationship matrix for parents and progeny. As the analysis was based on open-pollinated progeny from native patents, the additive relationship matrix used to estimate the additive genetic effects was modified to take into account a selfing rate of 10% for *E. pauciflora* (Gauli *et al.* 2014) and a base level of population inbreeding of 0.067 (Gauli *et al.* in press). This modification accounts for differences in the coefficient of relatedness (*r*) amongst sibs and between sibs and their parents in estimating additive genetic effects and followed the procedure of Dutkowski and Raymond (2001). This was implemented in ASReml using the !SELF and !FGEN options respectively. Similar adjustment of the coefficients of relatedness using an average outcrossing rate and inbreeding level have been used in other studies of eucalypts to estimate additive genetic variances from open

pollinated progenies (Apiolaza *et al.* 2005; Blackburn *et al.* 2013; Bush and Thumma 2013).

Following Hamilton *et al.* (2013), estimates of narrow-sense heritabilities within populations (h^2_{op}) were obtained for each trait from the open-pollinated families using variance components estimated from the univariate analysis as follows:

$$h^2_{op} = \frac{\sigma^2_{\mathrm{a}}}{\sigma^2_{\mathrm{a}} + \sigma^2_e}$$

where σ^2_{a} is the additive genetic variance within populations; σ^2_e is the residual variance derived from model 2. The significance of the heritability estimates was tested using Z tests, and the significance of σ^2_{a} was tested using a one-tailed likelihood ratio test (LRT, Gilmour *et al.* 2009).

Coefficients of additive variation (CV_A) were calculated as follows to compare the level of additive genetic variance in each trait independently of their means as follows:

$$CV_A = 100\frac{\sqrt{\sigma^2_a}}{\bar{x}}$$

where, σ^2_{a} is the additive genetic variance and $\bar{x}$ is the phenotypic mean of the trait.

The quantitative trait inbreeding coefficient (Q_{ST}) which is analogous to the molecular inbreeding coefficient F_{ST} (Chapter 3), was estimated as follows:

$$Q_{ST} = \frac{\sigma^2_p}{\sigma^2_p + 2\sigma^2_a}$$

where, σ^2_p is the population variance and σ^2_{a} is as defined above (Latta 1998). A comparison of Q_{ST} with the putatively neutral nuclear microsatellite differentiation F_{ST} was done using the maximum value of 0.07 obtained for 10 microsatellite loci for the progenies of the species (Chapter 3). A Q_{ST} value significantly greater than F_{ST} provides evidence of diversifying selection (Edelaar and Björklund 2011; Whitlock 2008), and this has been reported in several recent studies of population differentiation in eucalypts (Dutkowski and Potts 2012; Hamilton *et al.* 2013; O'Reilly-Wapstra *et al.* 2013).

Following Dutkowski and Potts (2012), the test was done by comparing likelihoods of the unconstrained model 2 to one where $Q_{ST} = F_{ST}$ using the constraint:

$$\frac{\sigma_a^2}{\sigma_p^2} = \frac{1 - F_{ST}}{2F_{ST}}$$

In the present case the ratio was 6.64, and this constraint was implemented in ASReml using the option !VCC. A one-tailed likelihood ratio test was then used to test the difference between the two models and thus whether $Q_{ST} > F_{ST}$. Variance components for estimating h_{op}^2 and Q_{ST} were obtained from univariate analyses, with their standard errors calculated using an expanded Taylor series (Gilmour *et al.* 2009).

The genetic correlations among traits were calculated using pair-wise bivariate analyses undertaken using a family model where the *tree* term in model 2 was replaced with the *family(population)* term. The covariance structures for each random term were fitted using the CORGH option of ASReml and parameter estimates generally unconstrained. Univariate estimates of variance components were used as starting values for parameter estimation, and where convergence problems were encountered convergence was usually achieved by fixing one or more of these values. This followed the approach widely used for open-pollinated families of eucalypts (Hamilton *et al.* 2013; Jordan *et al.* 1999) and the genetic correlations were estimated as:

$$r_{1,2} = \frac{\sigma_{1,2}}{\sqrt{\sigma_1^2\,\sigma_2^2}}$$

where, $r_{1,2}$ is the correlation between traits 1 and 2 at the defined genetic level (family within population [r_{family}] or at population [r_{pop}] level), $\sigma_{1,2}$ is the covariance between traits, σ_1^2 and σ_2^2 are the variance component for each traits (Jordan *et al.* 1999). Two-tailed likelihood ratio tests were conducted to determine if genetic correlations were significantly different from zero (Gilmour *et al.* 2009).

4.2.5 NIR data analysis

The variation amongst individuals in NIR spectra was summarised using principal components analysis undertaken with Unscrambler (version.10.0.1.). The first 25

principal components, that cumulatively explained 99.75 % of variation in the NIR spectral data, were used for subsequent analysis. Discriminant function analysis, vector fitting and spatial autocorrelation analysis were performed as described above for the morphometric analysis. Genetic correlations between the vectors derived from this analysis and the morphological and growth traits, were assessed using bivariate analyses, as described above. In this case, as only one seedling was assessed per family, the family residual for the NIR trait was fixed to zero.

4.3 Results

4.3.1 Genetic variation between populations

Twenty-three out of the 25 seedling traits were found to be significantly different among populations (Table 4.3). Vein angle at 4th node (VA4) and an aspect of the 11th node leaf shape (LWP LL11) were the only two traits that were not significant. Q_{ST} estimates for each trait ranged from 0.0 to 0.40, averaging 0.17 (Table 4.3). This large variation of Q_{ST} suggests that the various traits have been differentially affected by selection. The highest value was observed for relative lignotuber size (Lig size, 0.40), followed by number of nodes with lignotubers (LIGNO1, 0.39). Likelihood ratio test of the Q_{ST} values against putatively neutral F_{ST} showed 13 traits had significantly more differentiation among populations than expected (Table 4.3).

There was a significant but weak correlation of genetic-based Mahalanobis distances (calculated from the morphological and growth data) and the geographic distances amongst populations (Mantel r = 0.23, n = 37, P = 0.003). Populations within 27 km of each other showed stronger genetic relatedness than would be expected by chance alone (Fig. 4.2a). Beyond this distance there was no significant decrease in the correlation. A virtually identical pattern was observed with the Mahalanobis distances derived from the NIR data (Mantel r = 0.26, n = 37, P = 0.003; Fig. 4.2b).

Stronger Mantel correlations were found between the Mahalanobis distances in morphology between populations and their difference in (i) altitude (Mantel r = 0.66, P < 0.001), or (ii) overall climate as assessed by the 35 bioclimatic variables (standardised Euclidean distance matrix, Mantel r =0.47, P < 0.001), than with geographic distance. The major role of altitude and associated climatic variables in determining population

differentiation is evident from the major directions of variation in the discriminant space derived from both the morphology and NIR data sets. In the case of the morphological discriminant space, vector fitting using 38 variables, including 35 climatic variables representing temperature, moisture, precipitation and radiation, showed 33 climatic variables as well as altitude, latitude and longitude were significantly related to population differentiation. However, it was altitude and climatic variables, mainly related to temperature and moisture, which were associated closely with the major direction of population differentiation in seedling morphology/growth traits as defined by CV1 (39%) (Fig. 4.3a). This was particularly the case for mean maximum temperature of the warmest month (TMXWM), mean temperature of the warmest quarter (TWMQ), and mean moisture index of the warmest quarter (MIMWMQ) (Fig. 4.3a). This major direction of population differentiation was associated with decreasing altitude which was related to increasing temperature and decreasing moisture (Fig. 4.3a). Statistically significant population variation in the discriminant space was observed with latitude and longitude, but this variation was of minor significance and was in a different direction to the major variation explained by CV1 and CV2.

In the NIR discriminant space, 29 of the climatic variables were significantly related to population differentiation. However, in this case the degree of differentiation of populations along CV1 (17%) and CV2 (14%) were more similar, and association with climatic variables was more related to variation along CV2. Consistent with the morphological results, population differentiation in NIR spectra was mainly associated with altitude and variables reflecting differences in temperature and moisture, although one radiation variable was also highly significant (RRH) (Fig. 4.3b).

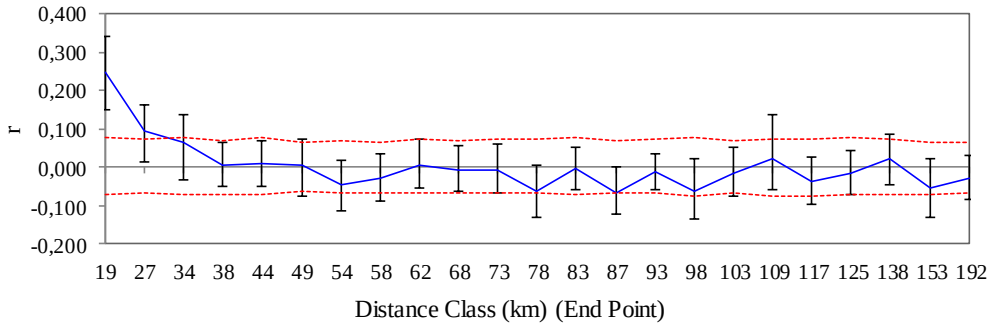
Spatial autocorrelation indicated a significant decline in Mahalanobis distance with increasing difference in altitude (Fig. 4.4a) and climate (data not shown) between sites. In the case of altitude, populations within 148 m of each other in altitude, exhibited above average genetic similarity, but thereafter there was a trend for decreasing genetic similarity with increasing altitudinal separation (Fig. 4.4a).

Table 4.3. Genetic parameters for the 25 seedling traits studied in Tasmanian *Eucalyptus pauciflora*. The table includes the trait code (see Table 4.2), overall trait mean, the F value for the differences between populations ($F_{36,\ 238}$) and its significance (Sig), the quantitative inbreeding coefficient (Q_{ST}) and its standard error (SE), the significance level for the one-tailed likelihood ratio test of the difference of Q_{ST} from the neutral marker maximum F_{ST} (LRT), the z value for the random variation between families within populations (z), the significance level for the one-tailed likelihood ratio test of the difference of the additive genetic variance component estimate from zero (sig), open-pollinated estimate of the narrow-sense heritability (h^2_{op}), and the within population coefficient of additive variance (CV_A). CV_A values are not presented for variables which were log transformed or where the additive variance was not significant. Significance levels are: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns = $P \geq 0.05$.

Traits	Mean	Between populations					Family(population)				
		$F_{36,238}$	Sig	Q_{ST}	SE	LRT	z	sig	h^2_{op}	SE	CV_A
HT1	44.32	1.58	*	0.04	0.03	ns	3.13	***	0.46	0.14	12.28
HT2	58.40	1.91	**	0.08	0.05	ns	1.88	*	0.32	0.15	10.23
LIGNO1	1.14	9.57	***	0.39	0.11	***	2.65	**	0.39	0.14	40.88
LIGNO2	1.80	9.37	***	0.31	0.08	***	3.72	***	0.57	0.14	35.12
STMD	5.16	2.45	***	0.08	0.04	ns	3.87	***	0.63	0.15	13.40
Lig size	0.80	10.86	***	0.40	0.11	***	2.91	***	0.39	0.13	25.18
Stem OG	1.28	4.06	***	0.13	0.04	*	4.63	***	0.73	0.15	38.76
Leaf colour	1.83	7.45	***	0.29	0.08	***	3.44	***	0.50	0.14	19.85
NNODES1	9.23	4.56	***	0.18	0.06	**	3.74	***	0.57	0.14	9.65
NNODES2	12.21	3.33	***	0.11	0.04	ns	4.01	***	0.70	0.15	12.86
PET2	0.63	4.2	***	0.18	0.07	**	3.28	***	0.45	0.14	16.78
ALT2	0.24	4.4	***	0.17	0.06	**	3.7	***	0.54	0.14	43.75
TWIST2	0.23	4.36	***	0.20	0.08	**	2.78	**	0.38	0.14	37.33
LL4	6.48	3.13	***	0.09	0.04	ns	4.87	***	0.75	0.14	14.60
LW LL4	0.48	3.37	***	0.13	0.05	ns	3.4	***	0.48	0.14	-
LWP LL4	0.42	1.83	**	0.07	0.05	ns	2.22	*	0.28	0.13	8.33
LA4	15.45	2.58	***	0.08	0.03	ns	4.34	***	0.64	0.14	24.91
PL4	0.06	3.13	***	0.22	0.13	*	1.37	ns	0.21	0.13	-
VA4	47.06	1.35	ns	0.02	0.03	ns	2.78	**	0.39	0.14	7.79
LL11	8.64	4.1	***	0.13	0.04	ns	4.51	***	0.77	0.15	16.22
LW LL11	0.44	1.98	***	0.04	0.02	ns	5.1	***	0.89	0.15	-
LWP LL11	0.37	0.72	ns	0.00	0.00	*	2.12	*	0.26	0.13	8.99
LA11	24.01	5.7	***	0.27	0.09	***	2.72	**	0.40	0.14	20.67
PL11	0.25	12.09	***	0.37	0.08	NC	4.39	***	0.71	0.15	-
VA11	33.75	3.92	***	0.30	0.17	**	1.25	ns	0.20	0.14	9.15

NC: did not converged

a) Morphological traits



b) NIR PCA

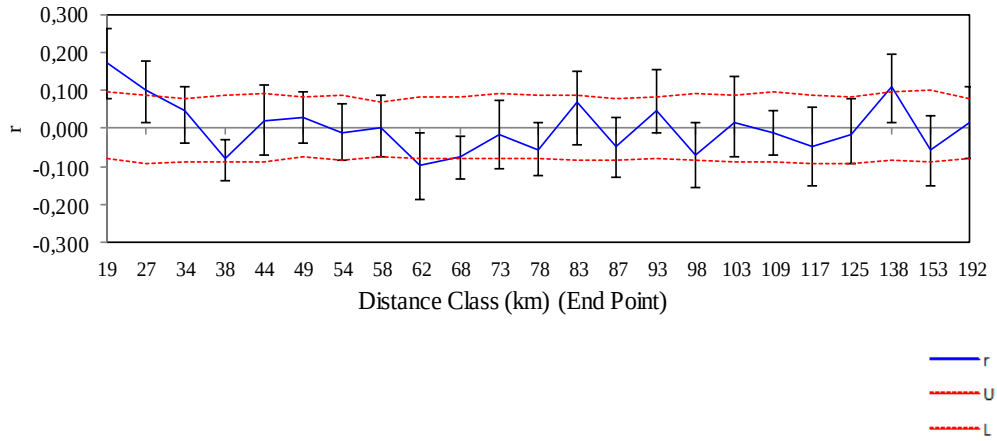
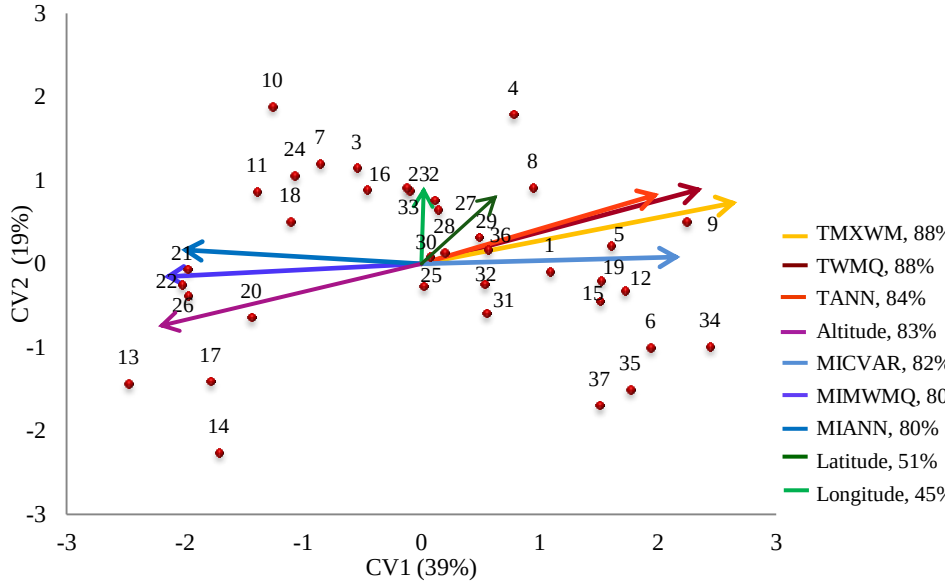


Fig. 4.2. Correlograms of *Eucalyptus pauciflora* populations from Tasmania based on the geographic distance and a) Mahalanobis's distances derived from an analysis of 25 morphological traits, and b) Euclidean distance matrix calculated from principal components derived from the leaf near-infrared spectral (NIR) data. *r* is the autocorrelation coefficient, upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of no spatial structure for the combined data set as determined by permutation using GENAlEX 6.501 (Peakall and Smouse 2006).

a) Morphological data



b) NIR data

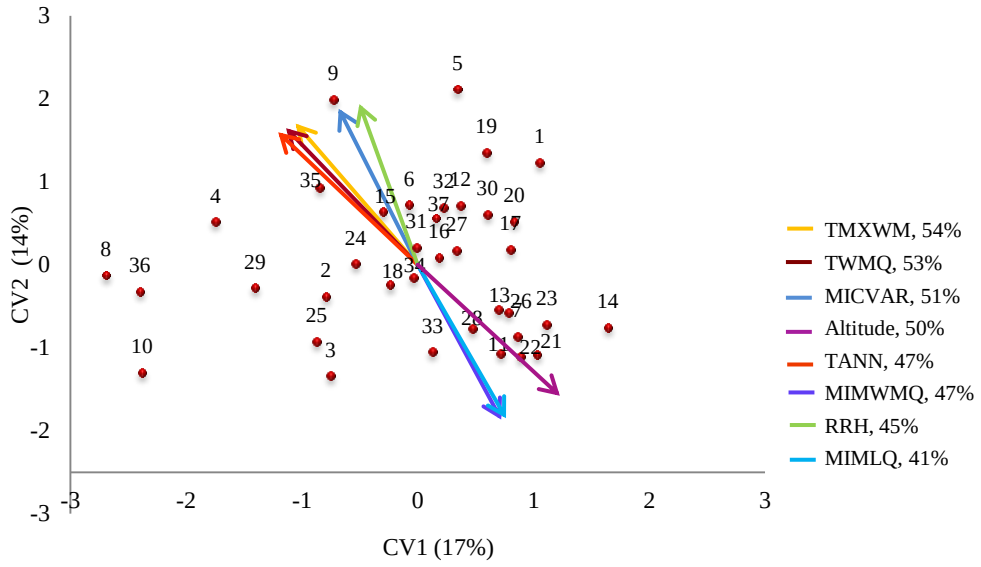
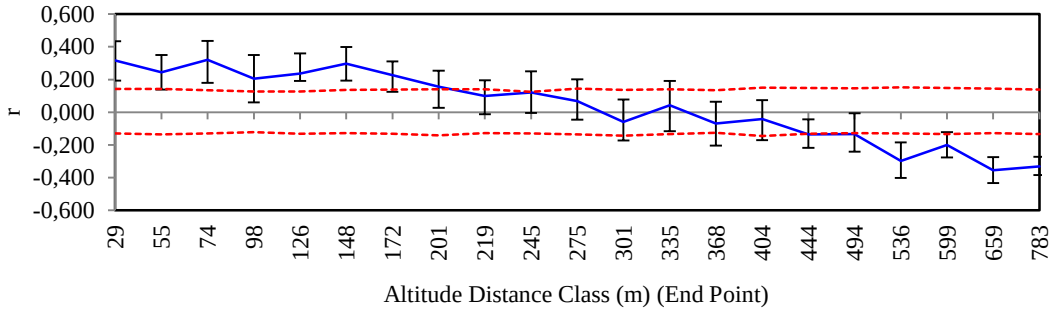
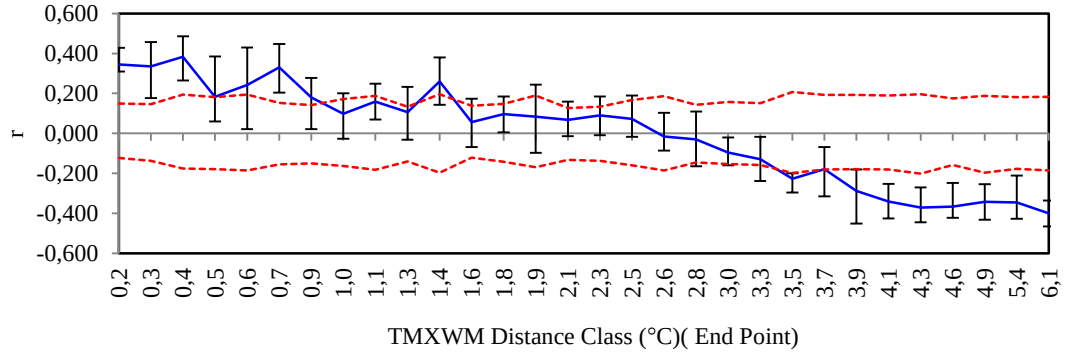


Fig. 4.3. Fitted climatic vectors, altitude, latitude and longitude (if significant) in a) the ordination of the *Eucalyptus pauciflora* population centroids along discriminant axes based on the morphological analysis, and b) the ordination of the *E. pauciflora* population centroids along discriminant axes based on 25 significant principal component analyses of the NIR spectra. Vectors indicate the magnitude and the direction of the population differentiation. Climatic variables plotted here are variables (out of 35 variables) that were most highly correlated ($P < 0.001$) and altitude, latitude and longitude if they are significant with population variation in the two-dimensional discriminant space. TMXWM = mean max temp of warmest month, TWMQ = mean temperature of the warmest quarter, TANN = mean annual temperature, MICVAR = coefficient of variation (moisture index seasonality), MIMWMQ = mean moisture index of warmest quarter, MIANN = mean annual moisture index, RRH = highest period of radiation, and MIMLQ = mean moisture index of lowest quarter.

a) Genetic distance vs altitude



b) Genetic distance vs TMXWM



c) Genetic distance vs MIMWMQ

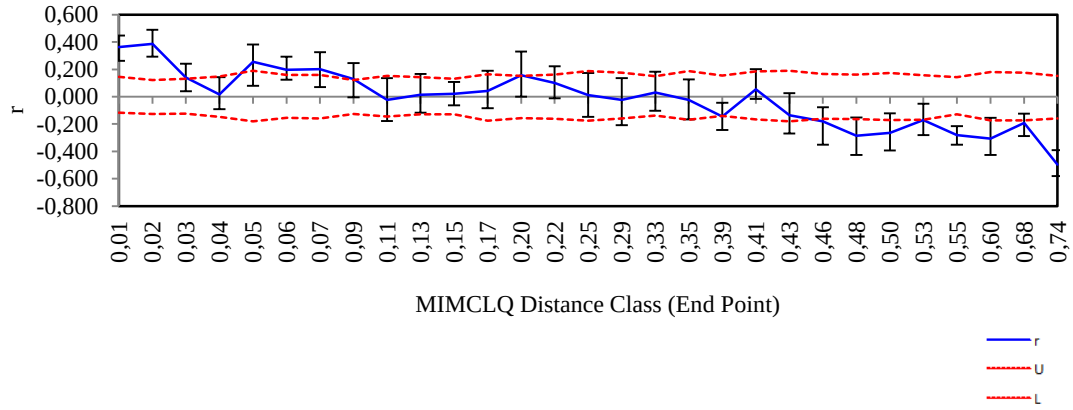


Fig. 4.4. Correlograms of *E. pauciflora* populations from Tasmania based on the morphological Mahalanobis's genetic distances and a) altitude (m), b) mean maximum temperature of the warmest month (°C) (TMXWM), and (c) mean moisture index of warmest monthly quarter (MIMWMQ). *r* is the autocorrelation coefficient, upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of no spatial structure for the combined data set as determined by the permutation.

In contrast to the non-linear trends observed with geographic distance, autocorrelation analysis of the population differences in altitude and the key temperature variable TMXWM and moisture index MIMWMQ revealed a more-or-less linear decline in morphological Mahalanobis distance between populations with increasing temperature and moisture index difference of the site of origin (Fig. 4.4b, Fig. 4.4c). This association is reflected in their highly significant Mantels tests (TMXWM: Mantel r = 0.64, $P < 0.001$; MIMCLQ: Mantel r = 0.51, $P < 0.001$). Populations with less than 1ºC difference in TMXWM were genetically more similar than average, and thereafter similarity declined.

Analysis of the relationship of morphological traits with altitude and climatic variables at the population origin at the univariate level yielded similar conclusions to the multivariate analysis. Thirteen of the 23 morphological traits that showed significant differences between populations were significantly associated with altitude (Table 4.4). Twenty-one of 23 traits were significantly associated with at least one climatic variable, but only 11 remained significant after accounting for multiple testing, all of which were significantly associated with altitude (Table 4.4). The climatic variable explaining most of the variation in many traits (seven) was the mean maximum temperature of the warmest month (TMXWM) and, while not the best predictor, this variable also explained significant variation in seven other traits (Table 4.4). The significant parallel responses to variation in this key climate variable were clearly evident for several traits. For instance, seedlings originating from hotter sites tended to have larger lignotubers (Fig. 4.5a), fewer oil glands on the stem (Fig. 4.5b), light coloured leaves (Fig. 4.5c), and later ontogenetic development of alternate leaves (Fig. 4.5d).

4.3.2 Genetic variation within populations

Highly significant within population genetic variation was observed in 23 of the 25 morphological traits, as evidenced by significant family within population variance (Table 4.3). The overall average of the individual narrow-sense heritability estimates was 0.50, with 21 of the 25 traits having estimates greater than 0.3. In terms of the growth traits, the highest heritabilities were evident for the number of nodes (NNODES 2, h^2_{op} = 0.70) and stem diameter (STMD = 0.63). Of the ontogenetic traits, leaf alternation showed the highest heritability (ALT2 = 0.54) and of the leaf morphological

Table 4.4. Association of functional traits with altitude and climatic variables of the site of origin for seedling traits from the Tasmanian *Eucalyptus pauciflora* populations. Population least square mean estimates were used for the regression analysis to test the effect of altitude and climatic variables. Only traits where population differences were statistically significant are shown (see Table 4.3).

Traits	Altitude				Best univariate regression against climatic variables				
	R^2	Relationship	F	*P*	R^2	Factor	Relationship	F	*P*
HT1	4%	negative	1.3	0.261	11%	TMNCM	positive	4.5	0.042^{ns}
HT2	0%	positive	0.2	0.681	16%	TCVAR	negative	6.8	0.014^{ns}
LIGNO1	62%	negative	58.1	<0.001	84%	TMXWM	positive	183.7	$<0.001^{***}$
LIGNO2	64%	negative	61.8	<0.001	81%	TMXWM	positive	146.6	$<0.001^{***}$
STMD	30%	negative	14.9	0.001	33%	TMXWM	positive	17.4	$<0.001^{*}$
Lig size	66%	negative	69.4	<0.001	82%	TMXWM	positive	157.9	$<0.001^{***}$
Stem OG	63%	positive	60.4	<0.001	69%	TMXWM	negative	77.4	$<0.001^{***}$
Leaf colour	68%	positive	74.2	<0.001	71%	TANN^{*-}	negative	85.2	$<0.001^{***}$
NNODES1	16%	negative	6.8	0.013	27%	MICVAR^{*+}	positive	12.6	0.001^{*}
NNODES2	0%	negative	0.0	0.897	7%	TIT	-	2.6	0.114^{ns}
PET2	27%	positive	13.1	0.001	37%	MIMCLQ^{*-}	positive	20.7	$<0.001^{**}$
ALT2	42%	positive	25.1	<0.001	51%	TMXWM	negative	36.3	$<0.001^{***}$
TWIST2	33%	positive	17.3	<0.001	49%	TMXWM	negative	33.9	$<0.001^{***}$
LL4	1%	negative	0.3	0.612	14%	MIMCLQ	positive	5.8	0.021^{ns}
LW LL4	1%	negative	0.2	0.663	17%	TSPAN	positive	7.0	0.012^{ns}
LA4	3%	negative	1.1	0.307	15%	RCVAR	positive	6.2	0.018^{ns}
LWP LL4	1%	positive	0.2	0.654	8%	RRDRYQ	-	3.3	0.08^{ns}
PL4	10%	positive	3.8	0.060	24%	MIMCLQ^{*-}	positive	11.2	0.002^{ns}
LL11	17%	negative	7.1	0.011	21%	TCLQ^{*+}	positive	9.2	0.005^{ns}
LW LL11	2%	positive	0.1	0.788	16%	RRCLQ	negative	6.6	0.014^{ns}
LA11	17%	negative	7.0	0.012	22%	TIT^{*+}	positive	10.1	0.003^{ns}
PL11	28%	positive	13.3	0.001	45%	MIANN^{*-}	positive	28.9	$<0.001^{***}$
VA11	0%	negative	0.1	0.771	22%	RRWETQ	negative	9.7	0.004^{ns}

TMNCM = mean minimum temp of the coldest month, TCVAR = coefficient of variation of temperature, TMXWM = mean max temp of warmest month, TANN = mean annual temperature, MICVAR = coefficient of variation of moisture index, TIT = isothermality, MIMCLQ = mean moisture index of coldest quarter, TCLQ = mean temp of the coldest three month period, TSPAN = diff coldest monthly mean min & warmest monthly max, RCVAR = coefficient of variation of rainfall, RRDRYQ = mean radiation with rainfall of the driest quarter, TMDR = mean diurnal range, MIANN = mean annual moisture index, RRWETQ = mean radiation with rainfall of the wettest quarter, RRCLQ = mean radiation with rainfall of the coldest quarter. In the 'Factor' column, where best climatic variable was other than TMXWM, $*^{-}/*^{+}$ indicate a significant negative or positive correlation was also observed with TMXWM. Significance levels shown as superscripts after the *P* values in the last column denote the dependent false discovery rate significance after adjusting for multiple comparisons with the 35 climatic variables tested (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P \geq 0.05$).

Table 4.5. Genetic correlations between seedling traits in *Eucalyptus pauciflora* at a) the population level, and b) the family within population level. Trait codes are explained in Table 4.2. Where multiple measurements were made of the same or similar trait only the most recent or most relevant trait is presented.

a) Population level

	Population										
Traits	STMD	Lig size	Stem OG	Leaf colour	NNODES2	PET2	ALT2	TWIST2	LL11	LW LL11	VA11
HT2	0.01 ns	-0.60*	0.19 ns	0.24 ns	-0.01 ns	0.33 ns	0.43 ns	0.48 ns	-0.20 ns	-0.36 ns	-0.23 ns
STMD		0.59**	-0.95***	-0.71***	-0.08 ns	-0.27 ns	-0.41 ns	-0.46 ns	0.25 ns	0.22 ns	0.14 ns
Lig size			-0.98***	-0.83***	-0.21 ns	-0.55**	-0.74***	-0.72***	0.53**	-0.23 ns	-0.18 ns
Stem OG				0.89***	0.04 ns	0.46 *	0.65**	0.66***	-0.47 *	0.21 ns	0.09 ns
Leaf colour					0.10 ns	0.49*	0.69***	0.54**	-0.56**	0.14 ns	-0.15 ns
NNODES2						-0.45 ns	-0.28 ns	-0.37 ns	-0.92***	0.02 ns	0.31 ns
PET2							0.92***	0.96***	0.03 ns	-0.28 ns	-0.29 ns
ALT2								0.97***	-0.04 ns	-0.40 ns	-0.36 ns
TWIST2									0.09 ns	-0.39 ns	-0.26 ns
LL11										-0.25 ns	-0.34 ns
LW LL11											0.69**

b) Family level

	Family										
Traits	STMD	Lig size	Stem OG	Leaf colour	NNODES2	PET2	ALT2	TWIST2	LL11	LW LL11	VA11
HT2	0.56*	0.35 ns	0.14 ns	-0.77**	0.69***	0.04 ns	0.49 ns	0.58**	-0.19 ns	-0.23 ns	-0.18 ns
STMD		0.34 ns	-0.13 ns	-0.89***	0.14 ns	0.02 ns	-0.00 ns	0.21 ns	0.25 ns	0.04 ns	-0.05 ns
Lig size			0.02 ns	-0.64**	0.32 ns	-0.33 ns	-0.06 ns	-0.08 ns	-0.01 ns	0.02 ns	-0.03 ns
Stem OG				0.17 ns	0.07 ns	-0.22 ns	0.02 ns	0.14 ns	-0.12 ns	-0.08 ns	-0.04 ns
Leaf colour					-0.58***	0.38 ns	-0.11 ns	-0.41 ns	-0.32 ns	0.18 ns	-0.21 ns
NNODES2						0.03 ns	0.49**	0.37*	-0.36 *	-0.14 ns	0.01 ns
PET2							0.34 ns	0.28 ns	0.03 ns	-0.02 ns	0.08 ns
ALT2								0.63**	-0.10 ns	-0.09 ns	-0.03 ns
TWIST2									0.24 ns	-0.23 ns	-0.14 ns
LL11										-0.64***	-0.53 ns
LW LL11											0.17 ns

traits the highest heritabilities were observed for relative leaf width (LW LL11 = 0.89) and leaf length (LL11 = 0.77). The lowest heritability was observed for vein angle (VA11 = 0.20). In terms of the relative amount of within population additive genetic variation (CV_A), the highest values was observed for the developmental trait leaf alternation (ALT2 = 43.8%). High CV_A also observed for three lignotuber traits (e.g. LIGNO1 = 40.9%, LIGNO2= 35.1%). Some other developmental traits also had high CV_A (e.g. TWIST2 = 37.3%) as well as one leaf area trait (e.g. LA11 = 20.7%) also had relatively high CV_A. Other leaf morphological traits and most growth traits had low values of CV_A.

4.3.3 Inter- and intra-population genetic correlation

Many of the developmental or allometric traits were highly inter-correlated as expected (data not shown) allowing reduction of the data set to 12 key traits describing different facets of variation in growth, morphology or seedling development (Table 4.5). In general age-age correlations of the same or equivalent traits were high ($r > 0.7$) at the family and population levels and thus only the latest measurement was included in the study of genetic correlations presented in Table 4.5. We similarly focused on a single measure of lignotuber development and three leaf morphological variables from the 11th node leaves. Seedling lamina length was highly genetically correlated with leaf area, both within and between populations ($r > 0.85$) and was the variable retained as an indicator of leaf size. The relative leaf width was retained as an indicator of leaf shape.

Many of the correlations among the 12 key traits were found to be significant at the population level (Table 4.5a). Lignotuber size (Lig size), stem diameter (STMD), oil glands (Stem OG) and leaf colour were significantly inter-correlated. Seedlings from populations with larger lignotubers had larger stem diameters, had less oil gland development on the stem and had light green leaves. At the population level, vector fitting showed that three of these traits were also significantly ($P < 0.001$) correlated with variation in leaf NIR spectra (CV1-2 from Fig. 4.3b analysis Lig size $r^2 = 0.52$; Stem OG $r^2 = 0.44$; leaf colour $r^2 = 0.38$). This trend was confirmed by significant population level correlation between leaf colour and CV1 (r_{pop}=0.54, $P < 0.01$) and CV2 (r_{pop}= -0.57, $P < 0.01$) derived from NIR data (Fig. 4.3b). However these correlations were not significant among families within populations (CV1 $r_{family} = 0.22$,

$P > 0.05$ and CV2 $r_{family} = 0.20$, $P > 0.05$). At the family within population level, lignotuber size was genetically independent of all these traits except leaf colour (Table 4.5b). Stem diameter and oil glands were also independent at the family level. In contrast, leaf colour appeared to be genetically related to the stem diameter and lignotuber size at the family level, with lighter green leaves being associated with larger lignotubers and stem diameter. Despite many significant positive (stem diameter [STDM], number of nodes expanded [NNODES2], the onset of leaf twisting [TWIST2]) and negative (leaf colour) correlations involving seedling height (HT2) at the family level, seedling height was only correlated with one trait, lignotuber size, at the population level, with seedlings from populations with larger lignotubers tending to be shorter.

The onset of early vegetative maturity in seedlings, as indicated by a greater proportion of nodes with petiolate (PET2), alternate (ALT2) and twisted leaves (TWIST2), was associated with smaller lignotubers (Lig size), more stem oil glands (Stem OG) and dark green leaves (Leaf colour) at the population level (Table 4.5a). In contrast, none of these genetic correlations were evident within populations (Table 4.5b). The developmental traits themselves were highly positively inter-correlated at the population level, but only leaf twisting (TWIST2) and leaf alternation (ALT2) was genetically correlated within populations. An increased number of nodes expanded (NODES2) were correlated with smaller leaf lamina lengths (LL11) at both within and among population levels. At the population level, leaf lamina length was correlated with lignotuber size (Lig size) , stem oil glands (Stem OG) and leaf colour in such a way that seedling with smaller leaf lamina lengths was associated with larger lignotuber size, smaller oil glands and lighter green leaves. None of these correlations were observed at the family level.

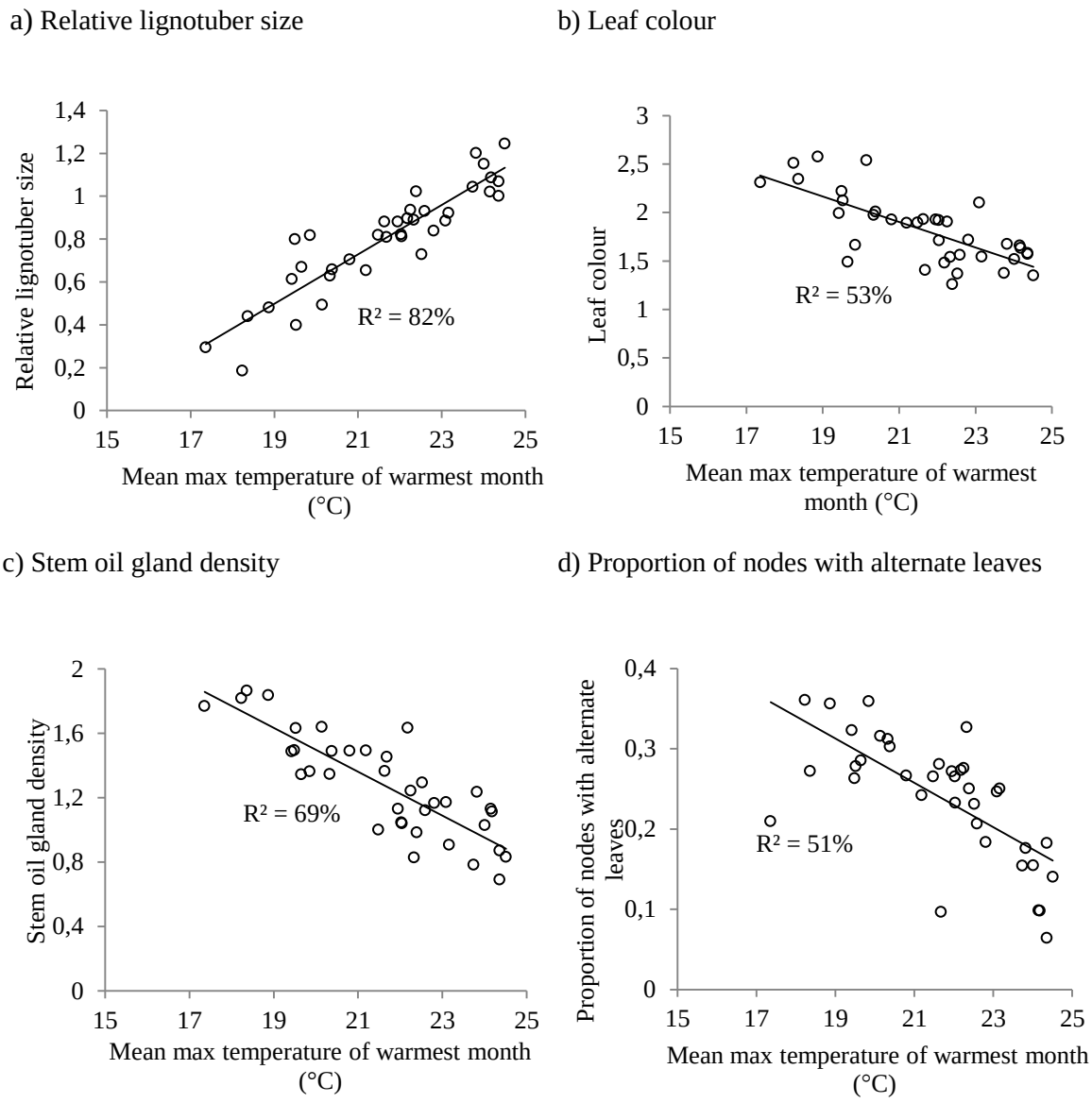


Fig. 4.5. The regression of the population least-square mean a) relative lignotuber diameter (Lig size), b) stem oil gland density (stem OG), c) leaf colour (light [1] to dark [3] green), and d) proportion of nodes with alternate leaves (PROP ALT), against mean maximum temperature of the warmest month (TMXWM) for *Eucalyptus pauciflora* populations in Tasmania. Traits are detailed in Table 4.2.

4.4 Discussion

4.4.1 Genetic variation between and within populations

A high level of genetic variation was observed both within and between Tasmanian populations of *E. pauciflora* for most traits studied. The level of quantitative inbreeding

coefficient (Q_{ST}) was high for many of the traits and likely to exceed neutral expectations in many cases. *E. pauciflora* is relatively widely distributed on the island and, consistent with other widespread species in Tasmania (*E. obliqua*: Bloomfield *et al.* 2011, *E. globulus*: Steane *et al.* 2006), there is likely to be few persistent historic barriers to pollen-mediated gene flow across the range of the species (Chapter 3). The maximum neutral marker estimate of F_{ST} for *E. pauciflora* is well below the average Q_{ST} and that of many traits such as those reflecting lignotuber size, leaf colour and developmental traits. While higher Q_{ST} values have been reported in species such as pine (Gonzalez-Martinez *et al.* 2002) and spruce (Mimura and Aitken 2007), our values were well within the range reported for oaks (Merilä and Crnokrak 2001) and other eucalypts (Dutkowski and Potts 2011). The Q_{ST} for lignotuber size (Q_{ST} = 0.40) in *E. pauciflora* was in the high range of Q_{ST} values reported in *Eucalyptus*, which was 0.42 for the adaptive trait of drought damage/resistance (Dutkowski and Potts 2012) and 0.71 for leaf cineole content in *E. globulus* (O'Reilly-Wapstra *et al.* 2013). These statistics suggest that selection may be driving population differentiation, at least for some of the traits studied.

4.4.2 Genetic differentiation is poorly associated with geographic distance

Consistent with this hypothesis, and despite considerable climatic variation within the native range of *E. pauciflora*, there was little relationship between population differentiation and geographic distance between populations beyond 27 km. Beyond this distance the degree of differentiation of populations was unrelated to their distance apart, arguing against limited gene flow and isolation by distance being responsible for broad-scale population differentiation in quantitative traits. Fine-scale localised relatedness may occur in eucalypt forest over several tree heights due to limited seed dispersal (i.e. family groups structure - Jones *et al.* 2007; Skabo *et al.* 1998), but in the present case it is the broader-scale population level affinities that are addressed. At this scale, the genetic affinities observed up to 27 km may reflect a distance over which broad-scale pollen dispersal defines a local population (Chapter 3). For example, using neutral molecular markers Bloomfield *et al.* (2011a) showed affinities of *E. obliqua* populations within 50-60 km, but little relationship with distance beyond. Yoeh *et al.* (2012) found that above-average genetic similarity in *E. globulus* to at least 40 km which they suggested reflected widespread pollen dispersal. However, as phenotypic rather than

neutral genetic similarity is addressed in the present study, coupled with the observed similarity in altitude and key climatic variables at the same localised spatial scale (data not presented), the possibility that this distance of 27 km also reflects the scale of adaptation to environmental homogeneity cannot be dismissed. In the case of the widespread *E. camaldulensis*, stronger, broad-scale spatial genetic structure was observed by Butcher *et al.* (2002, 2009) for both neutral marker and phenotypic similarity. This occurred both across, as well as within, major regions of the *E. camaldulensis* distribution, but was also confounded with strong associations with climatic variables. However, they found no association with altitude in *E. camaldulensis*, which contrasts with the present study where genetic-based difference between populations was more related with altitude than geographic distance apart. This might also be due to narrow ecological niche of the *E. camaldulensis* (Butcher et al. 2002). In the present case, this association with altitude is directly attributable to climatic similarity of the sites, particularly similarity in key climatic variables which change with altitude.

4.4.3 Genetic differentiation is strongly association with altitude and climate of origin

Strong association of altitude and climatic variables with various morphological traits have been reported in many tree species. Variation along altitudinal gradients well-known to confound changes in multiple facets of the environment (Körner 2007), and this is clearly evident in the present study where there are parallel changes in both temperature and moisture with altitude. Adaptation of morphological traits to altitude has been reported in diverse species including (*E. urophylla*: Tripiana *et al.* (2007); *Abies sachalinensis*: Ishizuka and Goto (2012), *Abies lasiocarpa*: Green (2005); *Quercus petraea*: Alberto *et al.* (2011); *Picea sitchensis*: Mimura and Aitken (2007)). Common garden trials showed altitudinal adaptation of phenology timing and the growth rate in beech, oak and the ash species (Vitasse *et al.* 2009). Height of all studied species decreased linearly with increasing altitude of the population origin (Vitasse *et al.* 2009). Adaptation to altitude is well documented in many eucalypt species (Kremer *et al.* 2014; Potts and Wiltshire 1997), including *E. pauciflora* (Slatyer 1978; Potts and Wiltshire 1997; Potts and Jackson 1986; Pryor 1956). The pioneering study of Pryor (1956) used progeny trials to show that the marked change in tree form and morphology with increasing altitude on mainland Australia was associated with significant genetic

based differences in seedling growth and morphology. Subsequent studies also revealed altitudinal differences in frost resistance may occur over short-distances, involving strong selection near the upper and lower (i.e. lower altitude frost hollows) tree lines (Harwood 1980; Harwood 1981). In our study, both multivariate and univariate approaches showed that the genetic based phenotypic variation amongst Tasmanian population of *E. pauciflora* were strongly associated with altitude and climate variation, with most traits showing strong correlation with at least one of the climatic variables studied. More than 50% of the traits showed association with the mean maximum temperature of the warmest month of the site of origin, suggesting an adaptive response. This is particularly evident for lignotuber related traits as well as for stem oil gland development and leaf colour.

Most eucalypt species including *E. pauciflora*, are heteroblastic, showing a distinct change in vegetative features (e.g. leaf morphology, phyllotaxy, orientation) from seedling to juvenile, intermediate and adult forms (Boland *et al.* 2002; Jordan *et al.* 1999; Lawrence *et al.* 2003; Loney *et al.* 2006; Wiltshire *et al.* 1998). The ontogenetic change may be accompanied by changes in foliar chemistry (Goodger *et al.* 2007; Goodger *et al.* 2006), susceptibility to herbivory (Loney *et al.* 2006) and growth rate (Jordan *et al.* 2000) and the adaptive significance of the change may vary between species or populations (Potts and Wiltshire 1997). Earlier vegetative maturation, expressed as earlier development of the petiolate, alternate and twisted leaves was attained in seedlings originating from sites with lower maximum temperatures and thus from higher altitudes. A parallel trend was also reported by Williams and Ladiges (1985), for mainland populations of *E. pauciflora* growing in a common environment trial, strongly supporting the hypothesis that this pattern is adaptive. In contast to our study, the reverse trend was observed in *E. nitida* (Shaw *et al.* 1984), in which higher altitude populations retained juvenile characteristics for longer.

Lignotubers have been described in many *Eucalyptus* species and they are often evident early in seedling development (Boland *et al.* 2002; Nicolle 2006a). They comprise protected vegetative buds, vascular tissue and food reserves and allow plants to regenerate after death of or damage to the main stem, following events such as fire, drought, frost or browsing (Potts and Pederick 2000). Large genetic-based differences in

lignotuber development have been observed between (Nicolle 2006a) and within *Eucalyptus* species (Ladiges 1974; Whittock *et al.* 2003). The number and size of lignotubers in *E. pauciflora* seedlings increased in populations originating from sites in Tasmania which experience higher maximum temperatures. Sites with higher maximum temperatures are expected to be more prone to drought and heat stress. Increased lignotuber development in populations from such sites might bestow greater ability to regenerate after death or damage to the main stem (Ladiges 1974; Whittock *et al.* 2003), arguing that the trend we observed is likely to reflect adaptation of *E. pauciflora* to hot, drought and fire prone environments.

The underlying cause of the leaf colour variation observed in *E. pauciflora*, from light to dark green, is at present unknown, but there is a clear trend for seedling leaves to become lighter green with increasing temperature and decreasing altitude of the site of origin. This colour change could be due to numerous factors including changes in leaf chemistry, physiology or surface properties. Higher photosynthetic activity and absorption was recorded on dark green leaves compared with those of lower intensity green in birch and oak species (Dillen *et al.* 2012). In the present case, the leaf colour variation was correlated at the population level with physicochemical variation of the leaf as assessed using NIR spectroscopy, but this variation was genetically independent at the family level suggesting parallel adaptation. The physicochemical variation (NIR spectra) may be indicative of variation in leaf defensive chemistry (McKiernan *et al.* 2012), but may also be indicative of other differences among populations in traits such as pigment and antioxidant composition (leaf colour) related to photoprotection (Close *et al.* 2007; García-Plazaola and Becerril 2000) and the inherent photosynthetic adaptation of *E. pauciflora*. In the latter case, a close phenotypic (Ferrar *et al.* 1989; Slatyer and Morrow 1977) and underlying genetic-based (Slatyer 1977a; Slatyer and Ferrar 1977a) adaptation of the photosynthetic physiology to increasing altitude has been reported. Adaptation has been shown to involve lower optimal temperatures for photosynthesis at higher altitudes in *E. pauciflora* (Slatyer and Ferrar 1977a) but may also involve mechanisms for photoprotection that have been reported in high altitude populations (García-Plazaola and Becerril 2000). Electron microscope scans of the leaf surface of single samples from 5 populations of seedlings classified as dark green and from 5 populations classified as light green revealed no clear consistent differences in

wax morphology (unpubl. data). In all cases the leaf surface waxes covering the juvenile leaves match the plate-like waxes previously reported for the *E. pauciflora* (Hallam and Chambers 1970; Li 1993), and is consistent with the reported structural glaucousness in *E. pauciflora* (Barber 1955).

The rugose stems of many eucalypt seedlings result from emergent oil glands ("verrucae"), the density of which in *E. pauciflora* increases with decreasing temperature and increasing altitude. It has been suggested (Ladiges 1984) that verrucae play a role in defence against herbivores (Ladiges 1984; Neish *et al.* 1995). If this was the case, our results would argue that herbivory pressure or the consequences of herbivore on plant fitness, increases as temperature decreases at higher altitudes. Increased insect herbivory has been recorded on eucalypts at higher altitudes in *E. gunnii* in Tasmania (Potts 1985), but the reverse trend has been reported for *E. pauciflora* forest on mainland Australia (Burdon and Chilvers 1974). An alternative explanation for the increasing density of stem oil glands at high altitudes might be introgression with the tree line species *E. coccifera*, which has highly rugose stems and hybridises with *E. pauciflora* in central regions of Tasmania (Williams and Potts 1996).

4.4.4 Correlation between traits and possible effect on adaptation

Many of the traits we studied showed correlated patterns of genetic variation at the population level and, from an evolutionary perspective, it is important to understand whether this is due to pleiotropy, genetic linkage or parallel evolution (Armbruster and Schwaegerle 1996). These alternatives can be resolved to some extent by comparing the degree to which genetic variation in various traits is correlated within, as opposed to between, populations (Armbruster and Schwaegerle 1996). High correlations amongst traits within populations may reflect either pleiotropy or tight linkage between underlying genes affecting multiple traits (Conner and Hartl 2004). Pleiotropy describes the genetic effect of a single gene on multiple phenotypic traits and, is the main persistent cause of such relationships and may result from allometric, developmental or biochemical relationships amongst the traits (Falconer 1989; Lynch and Walsh 1998). In the present case, while there is significant within-population genetic variation there are no significant genetic correlations within populations involving lignotuber size, stem diameter and oil glands. Within populations, these traits are also genetically

independent of the developmental traits describing the onset of early vegetative maturation as reflected in the development of petiolate, alternate and twisted leaves. This independence argues that the correlated patterns of population variation involving these traits are not due to pleiotropy but are the result of parallel evolution (selective covariance). As these traits are all significantly correlated with maximum temperature, our results argue that the adaptation to an increasingly more stressful hotter climate (as reflected by increasing TMXWM) will involve increasing lignotuber development, increasing stem diameter relative to height, decreasing stem oil gland density and a delayed onset of vegetative maturation in *E. pauciflora* seedlings and these traits are controlled by different genes.

In contrast, several other correlated patterns of trait variation amongst populations may simply reflect pleiotropic relationships amongst traits and, thus, correlated responses to selection. This is evident when the same high genetic correlations exist both between and within populations (Armbruster and Schwaegerle 1996). Such correlations were observed between: (i) the proportion of nodes with alternate leaves and the proportion of nodes with twisted leaves, and (ii) leaf colour and stem diameter and lignotuber size. Leaf alternation and twisting are signals of vegetative maturity and are likely to be part of a broader pleiotropic response associated with this ontogenetic transition (Wiltshire *et al.* 1998). However, the association of lighter green leaves with faster growth and larger lignotuber size is more difficult to explain. The possibility of tight linkage is unlikely when there is no significant within population genetic correlation between faster growth and lignotuber size, suggesting the possibility that the association is due to pleiotropy. While a pleiotropic relationship is difficult to explain, it could arise if the lighter green leaves absorb more radiation, which increases overall seedling productivity in the glasshouse environment, leading to increased resources being allocated to both stem and lignotuber growth. Before pleiotropy can be considered another possible explanation will have to be disproved, that the association might reflect a foliar response to depletion of the limited resources in the individual pots that were used to grow the seedling in the glasshouse. At the population level, such a mechanism would reflect a differential allocation of resources to increased stem diameter and lignotuber size at the expense of height growth. The patterns of genetic correlation

observed in the present study may reflect tradeoffs that potentially have an important role in evolution (Sgrò and Hoffmann 2004).

A key concern world-wide is whether forest tree populations are capable of responding to future climate change or not. The present study provides several lines of evidence for past adaptive response to key facets of climate. Standing genetic diversity or inter-population gene flow for adaptive traits is required for populations to adapt to environment change (Hancock *et al.* 2011; Kremer *et al.* 2012). Genetic variability for many traits among and within populations of *E. pauciflora* suggests the species clearly has evolutionary potential. This is particularly evident for several ecologically important traits (e.g. lignotuber size) which differ significantly among populations, and are strongly associated with climatic variables, especially mean maximum temperature of the warmest month. The autocorrelation analysis suggest that small changes in climate, such as a 1ºC change in the maximum temperature of the warmest month, are likely to lead to significant adaptive changes in the seedling phenotype. Significant additive genetic variation and heritability suggest that there is standing variation in populations of *E. pauciflora* to allow adaptation to environmental change. Indeed, the coefficients of additive variance for many of the adaptive seedling traits are high compared with those reported previously for forest trees, arguing for their evolvability (Marc and John 1998). However, understanding the response of individual traits to selection will depend upon not only the levels of genetic variability but their genetic correlations (Agrawal and Stinchcombe 2009; Guillaume 2011). Genetic correlations may be environment and/or population specific (Sgrò and Hoffmann 2004) and they can either facilitate or constrain evolution (Agrawal *et al.* 2010; Etterson and Shaw 2001). In order to constrain evolution very strong genetic correlations are required (Conner *et al.* 2011; Lynch and Walsh 1998), yet only a few genetic correlations amongst the traits identified as showing adaptive signals were observed to be very high in the present study. This argues that most of the correlated patterns of population differentiation observed are due to selective covariance (Armbruster and Schwaegerle 1996), and in most cases genetic correlations are unlikely to constrain future evolutionary change.

4.5 Conclusion

The present study has shown the close adaptive response of seedling morphology to climatic factors in *Eucalyptus pauciflora*. The close association of many traits with altitude at the site of origin and associated climatic variables, particularly the maximum temperature of the warmest month, suggests that population differentiation is to a large extent driven by variation in climate (especially temperature). Significant standing variation, high coefficient of additive variance and high heritability of ecologically important traits in *E. pauciflora* suggest that this species has the potential to adapt to environmental change from its standing variation. The absence of genetic correlation among many of the traits at the family level suggests many of these traits have potential to respond independently to selection.

4.6 Acknowledgements

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Chapter 5. Genetic variation in seedling performance in field trials of *Eucalyptus pauciflora*

Seedlings in the field trials: The top photo is of the Meadowbank trial site with 6 month old seedlings in the background showing trees just burnt by wildfire that occurred in early January 2013; the bottom photo is of 3 year old plants at the Dungrove trial site.

Abstract

The early-age performance of 281 *Eucalyptus pauciflora* families from 37 populations from the island of Tasmania and 15 population bulks from mainland Australia was studied in two Tasmanian field trials. These trials were established to test the extent of local adaptation and guide seed source choice for restoration purposes. There were significant population differences in early growth and susceptibility to drought and herbivory. Population differences in early performance appeared to reflect a trade-off between fast growth and herbivore susceptibility. Low altitude populations from warmer sites, initially grew faster but appeared to lose their advantage because of higher susceptibility to herbivory. Although height growth of populations was initially related to the climate at the site of origin, this relationship was insignificant by three years of age. Drought and high temperatures at one trial reshaped the fitness profile of the planting, selecting against populations originating from areas with more moisture. Integrating mating system parameters into the analysis of the two field trials revealed selfing resulted in significant inbreeding depression for height growth (33 % at the age of 3 years). However, outcrossing rate did not affect performance at the population level, arguing that population level processes other than inbreeding are more important to performance. At both trials the Tasmanian populations outperformed those from the mainland, suggesting that at this stage there is no support for seed translocations from mainland Australia for restoration projects in Tasmania. Within Tasmania there was some evidence for maladapation of some non-local populations at the more benign trial site, Dungrove, since a few non-local populations performed significantly worse than the local population. At the site subject to drought stress, many non-local island populations were more damaged than the nearest local population, but this did not translate to differences in survival and a year later many non-local populations were still superior in height growth. This study emphasises the complexities of the factors impacting on the success of tree establishment and the importance of establishing field trials in order to study the combined impact of abiotic and biotic factors, in addition to extreme selective events in determining the most favoured germplasm.

Keywords: *Eucalyptus pauciflora*, seedling growths, survival, genetic adaptation, field trials

5.1 Introduction

Restoration plantings are widely used to combat habitat fragmentation and tree decline (Close and Davidson 2002; Hobbs and Norton 1996; Ruiz-Jaen and Mitchell Aide 2005), and more recently to obtain carbon benefits (Bailey *et al.* 2013; Galatowitsch 2009). Seed source decisions are a key concern to ensure new populations became functional, persist and are resilient to environmental challenges (Broadhurst *et al.* 2008; McKay *et al.* 2005). The use of local genotypes is often viewed as a safe option and is widely practiced (Broadhurst *et al.* 2008; Jones and Monaco 2009; Kramer and Havens 2009; O'Brien *et al.* 2007). This view assumes that local populations are best adapted to the long term environment of the site thus less at risk of mal-adaptation (Bischoff *et al.* 2010; Hufford and Mazer 2003; McKay *et al.* 2005) and there is also less risk of deleterious effects from offsite gene flow (Byrne *et al.* 2011; Potts *et al.* 2003). Genetic contamination of local native populations through gene flow, and hybridization between local and non-local genotype may, for example, result in outbreeding depression or genetic swamping, which may have fitness cost (Jones 2013; Keller *et al.* 2000; Lenormand 2002). Local adaptation is well documented in tree species (Goto *et al.* 2011; Linhart and Grant 1996). However, whether the use of local genotypes for restoration is the best strategy is being increasingly questioned in the face of rapid global climate change, increasing globalisation of pests and diseases, as well as direct anthropogenic modification of the habitats being restored (Byrne *et al.* 2011; Sturrock *et al.* 2011; Weeks *et al.* 2011).

One of the main concerns raised over the use of local seed sources from fragmented or disturbed landscapes involve inbreeding and reduced genetic variability. Trees usually have mixed or outcross mating systems and often exhibit significant inbreeding depression (Sedgley and Griffin 1989). Fragmentation and isolation of trees may result in increased inbreeding in open-pollinated seed, resulting in inbreeding depression which may override local adaptation (Kramer and Havens 2009; Mimura *et al.* 2009). Several authors have also suggested the use of genotypes from fragmented landscapes may result in populations which are mal-adapted due to genetic drift (Lopez *et al.* 2009), or do not contain sufficient genetic variation to maintain long-term evolutionary potential (Sgrò *et al.* 2011). There is also the possibility that seed sources from remnant

trees in fragmented and disturbed landscapes may include increased levels of hybridisation (Field *et al.* 2008).

Seed sourcing for forest restoration needs to take into account future climate change as often these forests will be expected to survive hundreds of years. Climate models have predicted an increase in global temperature and changes in the hydrologic cycles, which are expected to have major impacts on the current distribution of many tree species (Kremer *et al.* 2014; McKenney *et al.* 2007), including eucalypts (Butt *et al.* 2013; Hughes *et al.* 1996). Changes in temperature and precipitation that go over the thresholds of physiological tolerance of tree species are likely to bring range shift in those species. Range shift from lower to higher altitude (Meshinev *et al.* 2000; Wardle and Coleman 1992) or to higher latitude (Grabherr *et al.* 1994) are expected as species respond by tracking the shifting climate (Walther *et al.* 2002). But whether species can actually disperse rapidly enough is one of the important questions in today's biological conservation.

Seed sourcing for restoration purposes is thus a complex issue, and there may be situations where non-local seed sources may be warranted and others where local are better. Choosing seed source involves balancing the need for sufficient genetic variation within populations with the need for long-term adaptive fitness. Identifying highly adapted populations with sufficient genetic diversity to allow future adaptation is a challenging issue, especially when aiming to restore the past system but at the same time building a system resilient to future changes (Montalvo *et al.* 1997; Sgrò *et al.* 2011).

This chapter uses *Eucalyptus pauciflora* field trials established for restoration purposes to address the extent to which populations studied in previous chapters are locally adapted. These populations differ in breeding system parameters such as outcrossing rate (Chapter 2) as well as quantitative seedling traits (Chapter 4), several of which showed evidence of climate adaptation, but populations differed little in neutral genetic diversity (Chapter 3). This chapter focuses on the tree establishment phase and aims to answer the following questions: i) is there genetic variation in fitness-related traits? ii) how much of the variation in these traits is a reflection of differences in inbreeding? iii)

are there climatic variables that can predict the genetic variation? iv) does inbreeding over-ride adaptive genetic variation? v) do mainland populations outperform Tasmanian populations? vi) does the local Tasmanian population perform best? and vii) if not, can we predict the best non- local seed source? Findings of this chapter will directly inform seed collection guidelines and restoration decisions.

5.2 Materials and methods

5.2.1 Genetic material

The seedlings used for the field trial were progeny grown from open-pollinated seed collected from 281 trees from 37 Tasmanian populations and seedlot bulks of 15 populations from mainland Australia. The Tasmanian populations were spread across the geographic and climatic range of *E. pauciflora* in Tasmania. Geographic information and the number of trees sampled in each of the 37 Tasmanian populations is same as given in Table 4.1, except one more tree was sampled from each of the Dungrove, Lake Rowallan, Longford, Nunamara, Rosarden and Waterhouse populations. To avoid sampling closely related individuals, a minimum distance of 100 m generally separated the sampled trees. This distance was more than double the average tree height and should transgress any family group structure in the forest (Jones *et al.* 2007; Skabo *et al.* 1998). Geographic coordinates and altitude were recorded for each tree and later these were used to estimate climatic variables for each population using ANUCLIM Version 6.1 (Xu and Hutchinson 2010) as detailed in Chapter 4. Bulked seedlots from the 15 mainland populations were included in the study, which were mainly from the southern part of the species range on mainland Australia (Table 5.1, Fig. 5.1). These bulked seedlots contained pooled seed from between 8 to 10 wild trees. ANUCLIM climatic variables for these bulked seedlots were derived from a single population coordinate.

5.2.2 Field trials

The study was undertaken on two genetics field trials situated in the Derwent Valley of Tasmania at Dungrove and Meadowbank (Fig. 5.2). The trials were part of a larger set of experiments within restoration plantings positioned to maximize the habitat connectivity within the fragmented landscapes (Bailey *et al.* 2013). Each planting comprised an area of 30 ha and represented differing climatic conditions.

The Dungrove field trial was established in 2010 at 569 m above sea level (latitude -42° 16' 29.31 " S, longitude 146° 53' 28.01" E), which is a mid-altitude site for Tasmanian *E. pauciflora*. The trial was on ex-agricultural land with remnant patches of native eucalypt trees. It was previously occupied by *E. pauciflora* woodland and surrounded by fragmented *E. pauciflora* and *E. tenuiramis* woodland. This adjacent woodland was the local seed source for the Dungrove population [25] included in the genetics trial. The trial was on a fine sandy loam soil derived from a Permian mudstone substrate. ANUCLIM (Version 6.1) climate parameters were predicted for the site. The mean annual rainfall was 624 mm, which is near the lower minimum of the species' precipitation range in Tasmania. The mean annual temperature was predicted to be 9.1°C, with maximum temperature of the warmest month of 21.5 °C, which are mid values for the Tasmanian range of the species.

The Meadowbank field trial was established in 2011 at 295 m above sea level (latitude -42° 38' 18.83" S, longitude 146° 49' 16.13" E). The trial was on previous pastureland and the area included a mix of bracken fern, acacias and remnant patches of native eucalypt trees of *E. viminalis* and *Acacia dealbata* and was surrounded by fragmented eucalypt woodland comprising *E. viminalis* and some *E. tenuiramis*. The trial was on sandstone substrate with a coarse sandy loam. The ANUCLIM predicted mean annual rainfall of the trial site was 748 mm and the mean annual temperature was 10.5°C, with maximum temperature of the warmest month of 22.7 °C. The closest *E. pauciflora* trees sampled to this site was the population at Curringa [35], 8.6 km away from the Meadowbank.

5.2.3 Site preparation

In early May 2010 (six months before plantings), the existing pasture vegetation at Dungrove was sprayed with a knockdown herbicide (Glyphosate plus Hasten™). Cultivation was undertaken a month after spraying with the site ripped and mounded using a bulldozer and savannah plough. The site was left fallow for three months after cultivation. A second application of the knockdown herbicide and also the residual herbicide (Simazine) were done in September 2010. Blocks were again cultivated to a fine tilth to the top of the mound two weeks prior to planting. The Dungrove trial was planted on October 2010.

In late August 2011, the edge of the Meadowbank trial was marked and the whole area of acacia thickets was raked. Cultivation was done a month before planting in September 2011 with the site ripped and mounded using a bulldozer and savannah plough, then sprayed with a combination of knockdown and residual herbicides (Glyphosate, Lontril™ and Simazine). The genetics trial was planted on November 1st 2011.

Both sites were fenced for protection from sheep (although incursion occurred later at Meadowbank), but plants at both sites were exposed to varying levels of marsupial browsing as well as to feral populations of the European fallow deer (*Dama dama*) (Dungrove only).

Table 5.1. *Eucalyptus pauciflora* populations from mainland Australia used for the study with their codes and geographic locations. Note that the Tasmanian populations used in the study are given in Table 4.1.

ID	Population	Latitude (°S)	Longitude (°E)	Altitude
38	Williamsons Springs	-33.46	149.85	1162
39	Mt Ginni	-35.21	148.46	803
40	Bugtown Hill	-35.52	148.44	1146
41	Tallaganda	-35.55	149.32	786
42	Bogong High Plains	-36.87	147.29	1618
43	Pastoria	-37.22	144.54	498
44	Mt William Grampians	-37.30	142.60	1101
45	Haddon	-37.59	143.72	392
46	Hillcrest	-37.62	143.64	427
47	Mt Baw Baw	-37.81	143.91	1560
48	Mount Mercer	-37.81	144.19	353
49	Durdidwarrah	-37.84	146.27	370
50	Meredith	-37.84	144.08	340
51	Mt Martha Gippsland	-38.29	145.01	146
52	Modewarre	-38.28	144.14	122

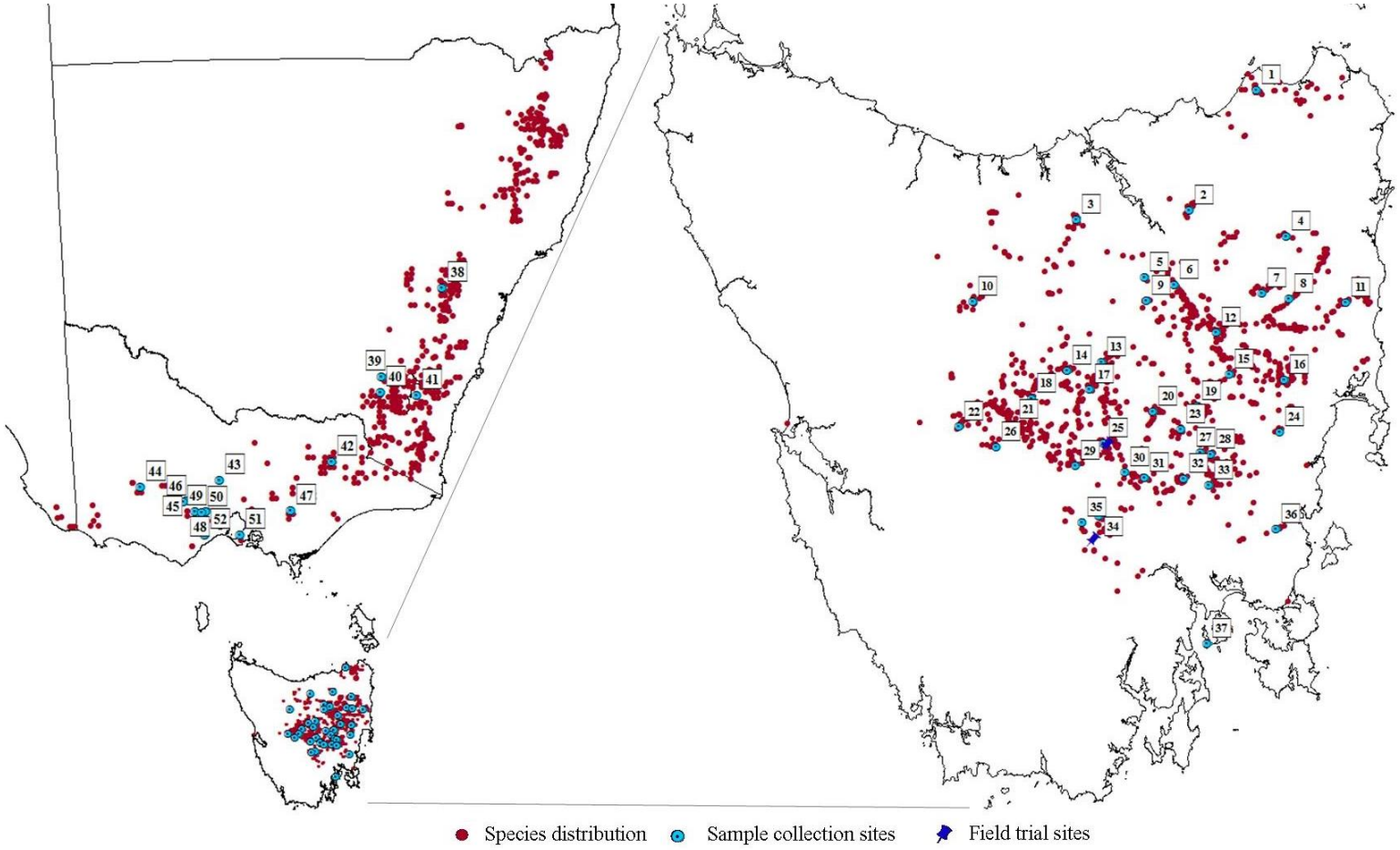

Fig. 5.1. Geographic distribution and seed collection sites of *E. pauciflora* in Australia and in Tasmania. Information on distribution of the *Eucalyptus pauciflora* on Mainland, Australia is based on Atlas of living Australia (http://www.ala.org.au/) and Tasmanian, Australia is based on the Williams and Potts (1996), Natural Values Atlas (www.naturalvaluesatlas.tas.gov.au) and additional records from the University of Tasmania. Population numbers correspond to those defined in Table 4.1 and Table 5.2.

5.2.4 Planting stock preparation and planting

Each collected seedlot was soaked in water overnight, drained, and then stratified at 4°C for 4 weeks. Each seedlot was then sown onto separate germination trays and allowed to germinate at room temperature in a commercial nursery. After 8 weeks (including stratification), germinants were pricked out into individual cells in HIKO™ (HV93) trays; where each tray contained 40 plants of one family (one tray per family). Tray positions were then randomised in an indoor growing area of a nursery and transferred outside after 10 weeks. Eight months after sowing for Dungrove and 16 months after sowing for Meadowbank, seedling were labelled with their family identification and then sorted into the experimental design in HIKO™ trays. Labelled seedlings were transported in boxes to the trial site and distributed to their designated replicates. Seedlings were handed to professional forestry planters in the sequence matching the design. Following Davidson and Close (2006) and Close and Davidson (2002), plants were planted with Potipuki No. 55 tree planters so that the root ball was approximately 2 cm below the soil surface to prevent desiccation. Soil was firmed down after planting to prevent air pockets between root ball and surrounding soil and to ensure seedlings were stable in the soil.

5.2.5 Trial design and layout

The genetic trials in each field site were established in an identical way. Eight replicates were established through each of the planting areas to obtain uniform planting areas (Fig. 5.2). In the case of Dungrove the replicates were not necessarily adjacent as some were interspersed through the planting area as other trials were planted at the same time in the area. At Meadowbank the replicates were more adjacent to one another. Each family (including mainland bulks) was randomized into a row X column design using CycDesigN 4.0 (Whitaker *et al.* 2002). There were 20 rows and 20 columns per replicate. Each replicate consisted of 400 treatments which were comprised of 281 Tasmanian families (each family represented by one seedling) and multiple seedlot positions from the 15 mainland populations. Mainland seedlots were represented by one to nine seedlings giving a total of 119 seedlings from mainland per replicate. The rows were 3 m apart and within rows seedlings were planted 2.5 m apart (i.e. between columns). Where replicates were not contiguous, they were surrounded on all sides by a line of *E. pauciflora* seedlings planted as a buffer.

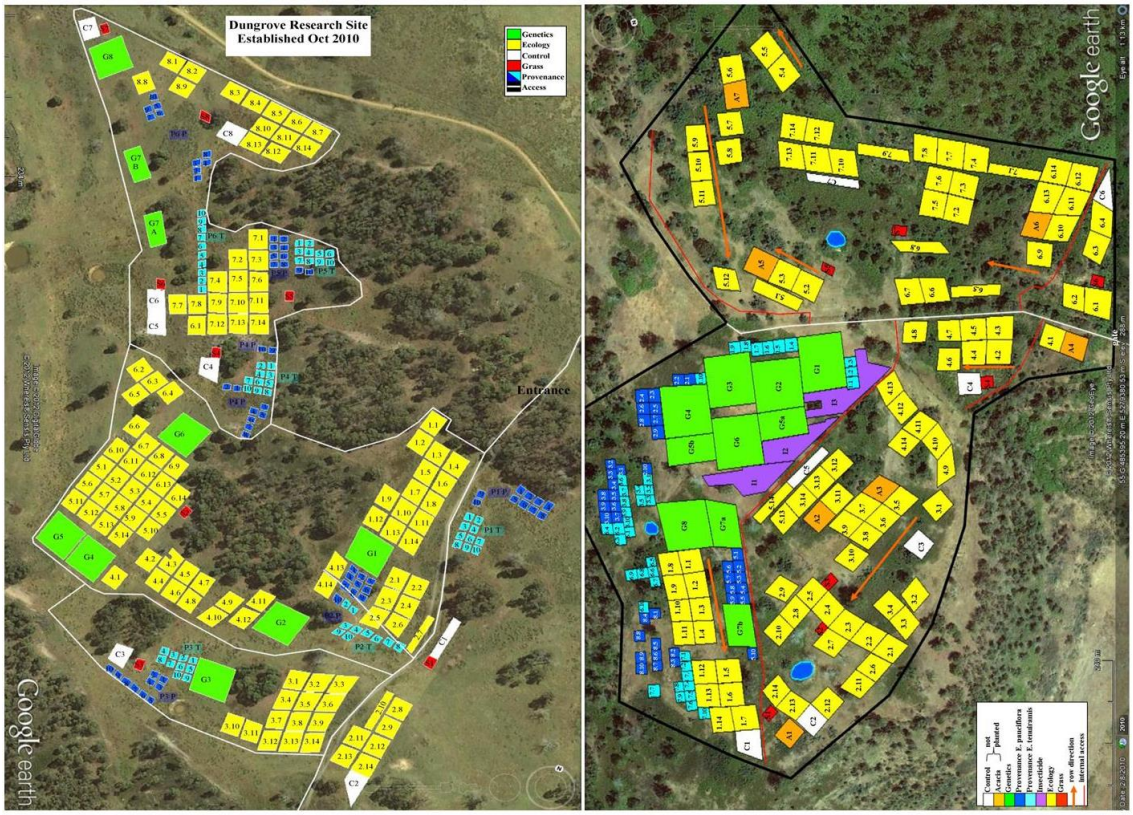

Fig. 5.2. Map of the experimental restoration planting sites situated in the Derwent Valley of Tasmania showing the disposition of the genetics field trials (green blocks) at, left- Dungrove field trial (Bailey *et al.* 2013) and right- Meadowbank field trial.

5.2.6 Trait assessment

In each field trial, survival and growth were monitored and scored on a regular basis, at least once every 6 months (Table 5.2, Fig. 5.3). Fitness related damage traits were recorded after the damage was evident. For instance insect damage or mammal damage was scored after outbreak of insect and browsing damage was observed or during routine growth assessments. At Dungrove, damage from frost was recorded after the trial site experienced significantly low temperatures in April 2011. The Meadowbank trial was affected by drought and high temperatures in the early January 2013. This stress event was accompanied with a week of severe bushfires and extreme hot temperature in southern Tasmania (Bureau of Meteorology 2013). Wildfire burnt the fence and some edge row plants of the Meadowbank trial but did not reach the plants in the genetics trial. The temperature peaked on 4th January 2013 with temperatures of 41.8°C (Bureau of Meteorology 2013). In order to assess the impact of this drought event on plant fitness, damage was assessed in 10 days after the event.

5.2.7 Statistical analysis

The data were analysed separately for each trial using linear mixed models. Quantitative traits were analysed using PROC MIXED and transformed where necessary to optimize the normality and homogeneity of variances. Binary traits (e.g. survival) were analysed using PROC GLIMMIX with a logit link function. In all cases, fixed terms were tested using the Walds F-test and least-square means (LSMs) estimated. These analyses were performed in SAS 9.2 **(**SAS Institute Inc. 2009).

To test for fixed differences between populations across a major latitudinal disjunction in the distribution of the species, Tasmania vs. mainland (i.e. state effect), the following model was fitted:

Y = *rep* + *row(rep)* + *col(rep)* + state + *population(state)* + *rep*population(state)* + *residual* ……model (I)

where, Y is an observation of the seedling trait, *rep* is replicate as a random effect, *row(rep)* represents the random row within replicate effect, *col(rep)* represents the random column within replicate effect, *population(state)* is the random effect of population within state and *rep*population(state)*is the random replicate by population within state interaction term. The random *population(state)* term was used as the error to test the fixed state effect.

To test for a difference between the bulk provenances samples from the mainland, the following model was fitted to the subset of mainland data with population as a fixed term:

Y = *rep* + *row(rep)* + *col(rep)* + population + *population*rep* +*residual*……model (II)

The random interaction between population and replicate (*population*rep)* was used as the error to test the fixed population effect as there was no pedigree information within these population bulks.

As the Tasmanian populations had families maintained separately, to increase the inference space the following model was fitted for quantitative traits using the subset of the data that comprised only the Tasmanian populations:

Y = rep + *row(rep)* + *col(rep)* + population + *family(population)* +*residual*…model (III)

The replicate and population terms were treated as fixed. The *family(population)* term is the random family within population effect and was used as the error term to test the fixed population effect. The significance of the *family(population)* variance component from zero was tested using a Z-test for quantitative traits (PROC MIXED) and likelihood ratio test for the binary traits (PROC GLIMMIX).

Binomial models were fitted using a logit link function. However, due to convergence issues, the random terms *row(rep)* and *col(rep)* were dropped from all binomial models. In cases where model convergence was still not possible, the binomial models were modified by either treating the replicate term as random (e.g. Tasmanian population analysis) and/or by dropping other random terms such as *family(population)*. When these analytical modification were done this is indicated in the results Tables. The least-square means for the population effects were estimated on the logit transformed scale as well as following back transformation to the original scale, to express as a proportion, using ILINK option in PROC GLIMMIX in SAS 9.2 (SAS Institute Inc. 2009). Comparisons of all populations with the closest local population sample for each trial site were undertaken using pair-wise contrasts specifying the specific local population as a control.

To test for a relationship between the population least-squares means for each trait with: (i) population altitude, (ii) the 35 ANUCLIM climatic variables, (iii) population-level outcrossing rate (t_m from Chapter 2), and (iv) biparental inbreeding estimates (t_m-t_s from Chapter 2); a univariate analysis of covariance was undertaken using PROC GLM of SAS. To account for multiple testing with climate variables, probabilities for each trait were adjusted for a dependent false discovery rate using PROC MULTITEST in SAS 9.2 (SAS Institute Inc. 2009). This analysis was used to identify the best significant predictor of the variation in population least-square means. The back-transformed least-square means to proportions were used for the binary traits. For key variables where *a priori* tests were warranted (e.g. altitude, mean maximum temperature of the warmest month (TMXWM) and the mating system parameters t_m and t_m-t_s) univariate regression

analysis was also undertaken using PROC REG of SAS. In addition, forward stepwise multiple regression analysis was undertaken using the full set of 35 climate and the two mating system parameters (t_m and t_m-t_s) to determine whether population variation in each trait was better modelled with more than one variable.

Fig. 5.3. Damage traits assessed in the field trials, a) a *Perperus malevolens* adult insect, causing damage to the tip of the seedlings, b) damage caused by *Perperus malevolens* insect to the seedings (tipdam_apr12), c) damage caused by an unknown insect (ins_nov10), d) seedling with 40% of the frost damage (frost_apr11), e) a seedling with drought damage and resistant to the drought (drought_jan13), and f) a seedling defoliated due to deer damage (deer_may13). (photos c,d,e,f from Paul Tilyard)

Table 5.2. Growth, survival and damage traits scored in field trials of *Eucalyptus pauciflora* for quantitative analysis. The table contains the description of the traits measured, age at which the traits were scored, codes used for each trait in this chapter, and type of trait and transformation used for analysis.

Description	Code	Assessment age after plantings	Trait type	Transformation
Dungrove trial site				
Height (cm)	ht_nov10	1 month	quantitative	
Height (cm)	ht_july11	9 months	quantitative	
Height (cm)	ht_may12	19 months	quantitative	
Height (cm)	ht_nov12	24 months	quantitative	
Height (cm)	ht_may13	31 months	quantitative	
Height (cm)	ht_nov13	36 months	quantitative	
Insect damage (%)	ins_nov10	1 month	quantitative	$\sqrt{}$Ins_nov10
Insect damage (%)	ins_dec11	14 months	quantitative	$\sqrt{}$Ins_dec11
Tip damage by *Perperus malevolens* insect (%)	tipdam_apr12	18 months	binary	tip damage 0% = 0; tip damage > 0% = 1
Frost damage (%)	frost_apr11	6 months	quantitative	$\sqrt[4]{}$Fr_dam
Survival (alive or not alive)	survival_july11	9 months	binary	
Survival (alive or not alive)	survival_may12	19 months	binary	
Survival (alive or not alive)	survival_nov12	24 months	binary	
Survival (alive or not alive)	Survival_may13	31 months	binary	
Survival (alive or not alive)	survival_nov13	36 months	binary	
Deer damage (0 = absence, 1 = damage)	deer_nov12	24 months	binary	
Deer damage (0 = absence, 1 = minor damage, 2 = severe damage)	deer_may13	31 months	binary	deer damage < 1 = 0; deer damage ≥ 1 = 1
Browsing damage (0 = absence,1 = minor damage, 2 = severe damage)	brows_nov13	36 months	binary	browsing < 1 = 0; browsing ≥ 1 = 1
Meadowbank trial site				
Height (cm)	ht0_nov11	1 month	quantitative	
Height (cm)	ht1_may12	7 months	quantitative	
Height (cm)	ht2_nov12	12 months	quantitative	
Height (cm)	ht3_jun13	20 months	quantitative	
Survival (alive or not alive)	survival_may12	7 months	binary	
Survival (alive or not alive)	survival_nov12	12 months	binary	
Survival (alive or not alive)	survival_jan13	15 months	binary	
Survival (alive or not alive)	survival_jun13	20 months	binary	
Drought damage (%)	drought_jan13	15 months	binary	drought damage < 5% = 0; drought damage ≥ 5% = 1
Browsing damage (%)	brows_nov12	12 months	binary	browsing < 1 = 0; browsing ≥ 1 = 1
Browsing damage (%)	brows_jun13	20 months	binary	browsing ≤ 95 = 0; browsing > 95 = 1

Inbreeding depression was estimated directly from the covariate coefficient (slope) derived from fitting the family level outcrossing rates as a covariate in an individual tree mixed model. This coefficient directly estimated inbreeding depression due to selfing (t_m = 0) which is expressed as a percentage of that expected under full outcrossing (t_m = 1), using the equation %ID = 100* slope/(intercept + slope), which equates to the standard expression for inbreeding depression: %ID =100*[(outx-self)/outx] (Hardner and Potts 1995). These values were estimated by fitting an individual tree mixed model which accounts for an average species wide-estimate of outcrossing in the coefficient of relatedness used in the estimation of additive genetic effects as used in Chapter 4. This was implemented with a restricted maximum likelihood (REML) model using an average information REML algorithm (Gilmour *et al.* 1995) implemented using ASReml 3.0 (Gilmour *et al.* 2009). The individual tree mixed model fitted was:

$$Y = rep + t_m + row(rep) + col(rep) + population + tree + residual \quad \text{.....model (IV)}$$

where, Y is an observation of the seedling trait, rep is the fixed replicate effect, t_m is the family-level outcrossing rate (Chapter 2) fitted as a covariate, *row(rep)* represents the random row within replicate effect, *col(rep)* represents the random column within replicate effect, *population* is the random population effect and *tree* is the random additive genetic effect for each seedling and *residual* is the random residual variation.

5.3 Results

5.3.1 Tasmania versus mainland populations

There was a highly significant difference ($P < 0.001$) between mainland populations and the Tasmanian population in all height measurements up to the age of 36 months (Table 5.3, Fig. 5.4). On average the Tasmanian populations grew better than the mainland populations and this trend was evident in both trial sites (Dungrove: ht_nov13 mainland 85.1 cm ± 9.92 [lsmean ± se], Tasmania 112.4 ± 9.40; Meadowbank: ht3_jun13 mainland 36 ± 2.75; Tasmania 45.8 ± 2.18). Initially (up to 19 months after planting) there was no significant difference in survival rate among Tasmanian and mainland populations at Dungrove site, but after 24 months a significant difference was evident, with greater survival of the Tasmanian populations (mainland 65.7 % ±5.86, Tasmania 84.2 % ± 5.48). No significant difference in survival between Tasmanian and mainland populations were evident over the 20 months of assessment at Meadowbank, despite

differences in height growth and the trial being affected by drought and high temperatures in January 2013. The foliage damage recorded on plants following this complex stress event was not significantly different between Tasmanian and mainland populations (drought_jan13; Table 5.3).

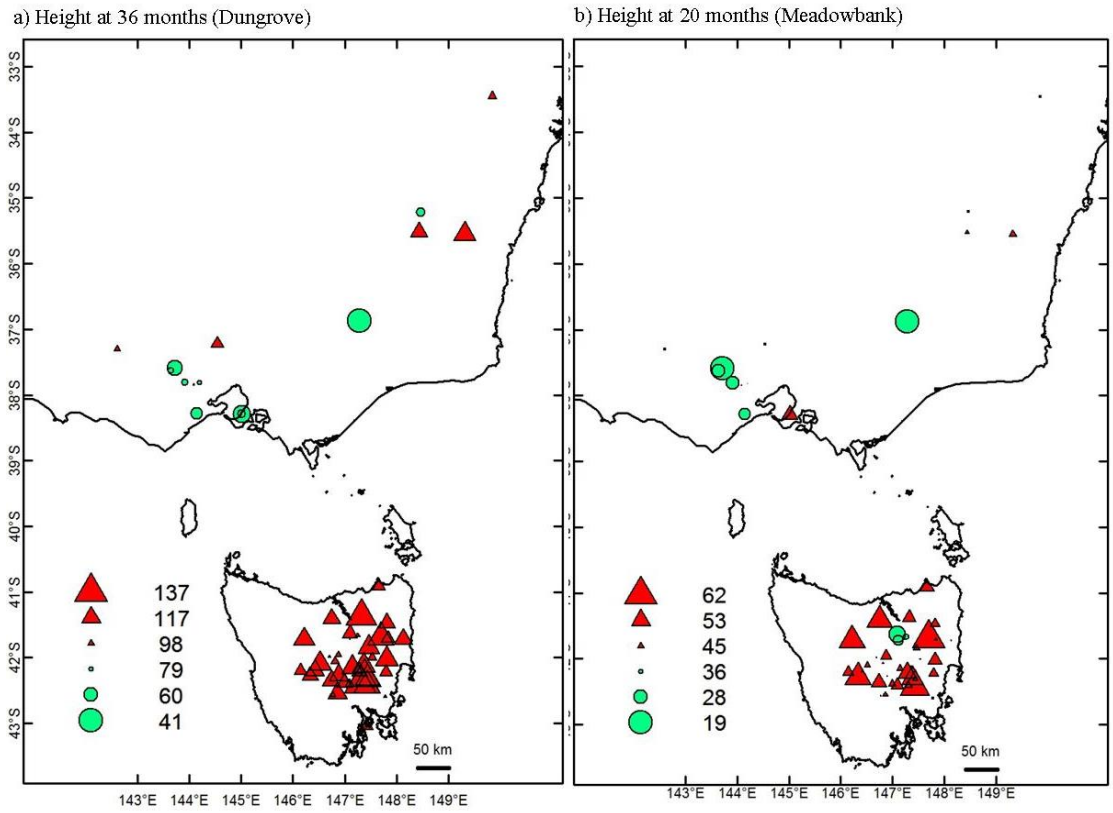

Fig. 5.4. Geographic variation of least square means of the last height measurements of the mainland and Tasmanian populations of *E. pauciflora* at trial sites: a) height at 36 months at Dungrove (cm) (ht_nov13), and b) height at 20 months at Meadowbank (cm) (ht3_june13). The larger the red triangle the greater is height of the plant and the larger the green triangle smaller the height. Note that Tasmanian populations are growing better than the mainland samples at both trial sites.

At Dungrove, there were no significant differences between the Tasmanian and mainland populations in early insect damage (ins_nov10) and deer browsing damage (deer_nov12), however, insect damage and deer damage at the later age were significantly different (Table 5.3). Insect damage (ins_dec11: mainland back transformed lsmean % damage 1.14, Tasmania 1.46; tipdam_apr12 proportion: mainland 0.20 ± 0.01 [lsmean ± se], Tasmania 0.27 ± 0.01) and the proportion of trees

damaged by deer (deer_may13 proportion: mainland 0.14 ± 0.01, Tasmania 0.24 ± 0.01) was greater on the faster growing Tasmanian populations. Despite no significant difference in early mammal browsing at Meadowbank, browsing mainly by sheep at 20 months was significantly different between Tasmanian and mainland populations. Unlike the previous trend observed at Dungrove, browsing was slightly greater on mainland than the Tasmanian populations (brows_jun13 proportion: mainland 0.90 ± 0.02; Tasmania 0.93 ± 0.01). Following the mild frost event at Dungrove, where leaf necrosis was observed on 40 % of the plants, Tasmanian populations were observed to be significantly less damaged than the mainland populations (frost_apr11; $P < 0.05$).

5.3.2 Genetic variation among populations within Tasmania and within the mainland

All height measurements at both Dungrove and Meadowbank showed highly significant genetic variation among the Tasmanian populations (Table 5.3). The geographic distribution of the faster growing populations changed with age (Fig. 5.5). At Dungrove the population with the greatest plant height at 36 months was the Oatlands [27] population and the tallest at Meadowbank after 20 months was the Rossarden [7] population. The Tasmanian populations did not differ in their survival at Meadowbank. However, significant differences were evident at age 31 months at Dungrove, but not in the subsequent assessment at 3 years. At Dungrove, the initial unidentified insect damage observed 1 month after planting was significantly different between populations ($P < 0.001$), but damage assessed by insects a year later did not differ significantly between populations, which could be caused by the same insects type or a different insect. Nevertheless, tip damage assessed following a weevil outbreak (*Perperus malevolens*) at 18 months of age, showed significant population differences (Table 5.3; Fig. 5.5). The deer damage observed on plants at Dungrove appeared to be mainly a result of rubbing their heads on the stems during antler shedding. While initially deer damage was significantly different among the Tasmanian *E. pauciflora* populations (deer_nov12; $P < 0.001$), the population differences were not significant 6 months later (deer_may13; $P > 0.05$). However, foliage browsing by unidentified mammals (probably mainly native marsupials) at Dungrove, 3 years after planting, was significantly different among populations (brows_nov13; $P < 0.001$). The recorded browsing at Meadowbank prior to the drought event was not significantly different

Table 5.3. Genetic parameters for the seedling traits of Tasmanian and mainland *Eucalyptus pauciflora* measured at the Dungrove and Meadowbank trial sites. The table includes the trait code (see Table 5.2), the F value and degrees of freedom for the differences between Tasmanian and mainland populations ($F_{1, 50}$) and its significance (sig), the difference between mainland populations ($F_{14, 98}$) and its significance, difference between Tasmanian populations ($F_{36, 238}$) and its significance and Z value for the random variation between families within populations (z) for quantitative traits or chi square likelihood ratio test (Chi LRT) for binary traits and its significance level. Significance levels are: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $P \geq 0.05$.

Traits	Assessment age (after plantings)	Tasmanian vs Mainland		Mainland Populations		Tasmania			
						Populations		Family(population)	
		$F_{1,50}$	sig	$F_{14,98}$	sig	$F_{36,238}$	sig	z/Chi LRT	sig
Dungrove trial site									
ht_nov10	1 month	6.1	*	35.5	***	4.3	***	8.4	**
ht_july11	9 months	15.1	***	6.0	***	4.1	***	3.7	***
ht_may12	19 months	36.8	***	7.0	***	5.5	***	1.2	ns
ht_nov12	24 months	36.7	***	6.0	***	3.8	***	1.1	ns
ht_may13	31 months	38.8	***	9.5	***	3.1	***	1.3	ns
ht_nov13	36 months	33.5	***	7.8	***	2.7	***	0.6	ns
survival_july11	9 months	0.1	ns	0.42 [b]	ns	0.4 [d]	ns	NC	NC
survival_may12	19 months	3.0	ns	2.23	**	1.0	ns	0.9	ns
survival_nov12	24 months	10.5	**	3.8	***	1.0	ns	0.8	ns
survival_may13	31 months	4.8	*	3.0 [b]	***	1.5 [b]	*	NC	NC
survival_nov13	36 months	7.0	*	5.9	***	1.2	ns	1.5	ns
ins_nov10	1 month	0.0	ns	5.4	***	4.4	***	8.4	***
ins_dec11	14 months	6.9	*	1.5	ns	1.3	ns	1.2	ns
tipdam_apr12	18 months	10.3 [a]	**	0.8	ns	2.2	***	0.0	ns
frost_apr11	6 months	6.8	*	8.9	***	5.3	***	3.3	***
deer_nov12	24 months	6.8 [b]	ns	1.95 [b]	*	2.0 [b]	***	NC	NC
deer_may13	31 months	27.6 [b]	***	1.48 [b]	ns	1.2	ns	2.8	*
brows_nov13	36 months	3.2	ns	4.1	***	2.8 [b]	***	0.2	ns
Meadowbank trial site									
ht0_nov11	1 month	19.1	***	45.0	***	3.5	***	8.2	***
ht1_may12	7 months	33.3	***	3.7	***	2.5	***	2.9	**
ht2_nov12	12 months	21.5	***	4.3	***	4.5	***	2.1	*
ht3_jun13	20 months	16.0	***	3.9	***	3.6	***	0.8	ns
survival_may12	7 months	0.0	ns	1.73 [b]	*	1.0	ns	1.2	ns
survival_nov12	12 months	1.3	ns	2.48 [b]	**	0.9	ns	1.0	ns
survival_jan13	15 months	1.7	ns	2.58 [b]	**	0.9	ns	1.4	ns
survival_jun13	20 months	2.7	ns	0.72 [b]	ns	0.9	ns	1.4	ns
drought_jan13	15 months	0.9	ns	1.1 [a]	ns	2.0	**	0.0	ns
brows_nov12	12 months	0.1	ns	3.2 [b]	***	1.2	ns	0.0	ns
brows_jun13	20 months	10.3 [c]	**	1.3 [a]	ns	2.9	***	0.4	ns

Note for convergence of some binary models, it was necessary to drop some of the random terms from the model. Modification on the model is denoted as: [a] dropping the rep term; [b] dropping all random terms; [c] dropping random population*replicate interaction error term from the model and [d] dropping random family(population) term. Model terms are detailed in the methods. NC = not converged.

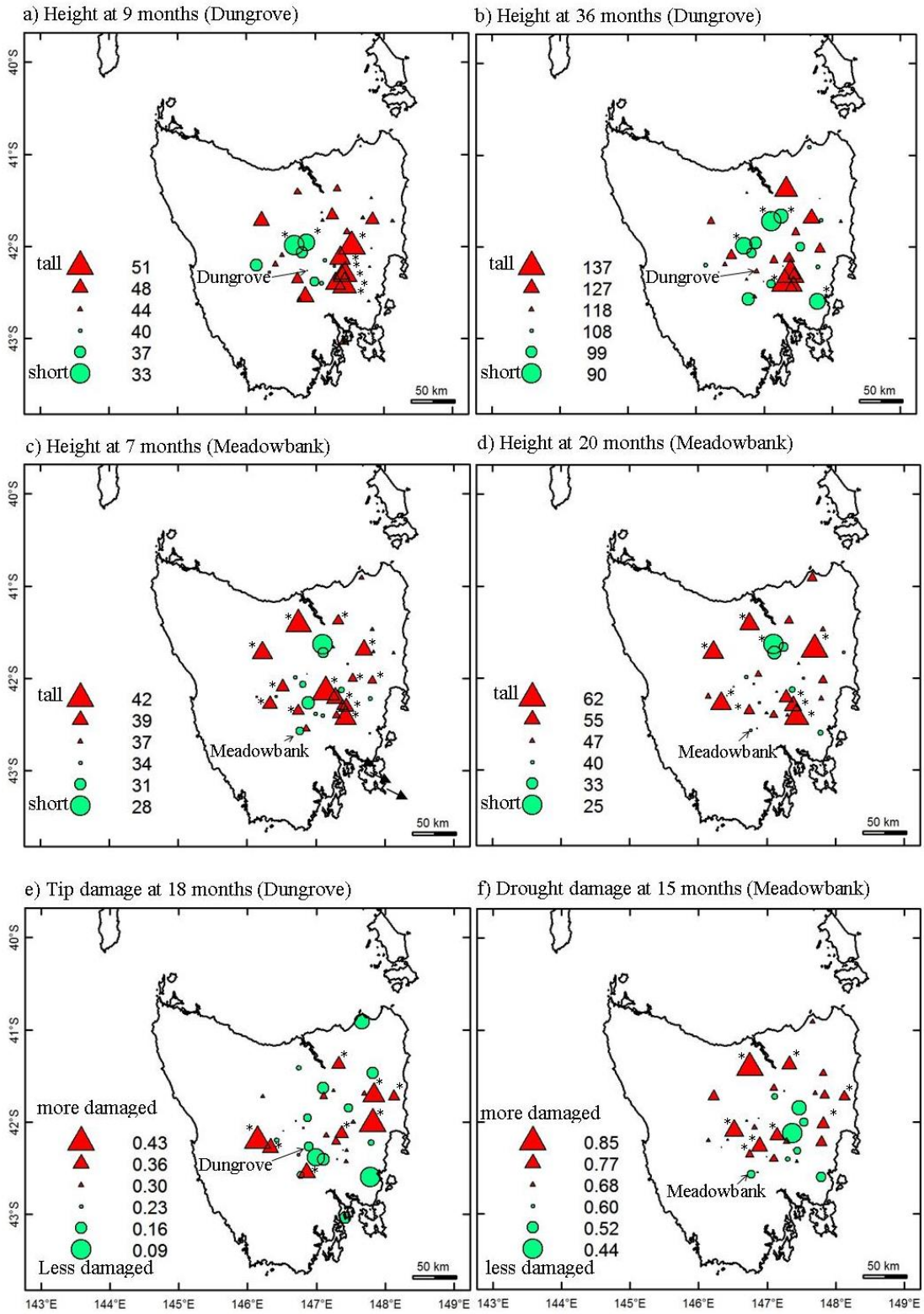

Fig. 5.5. Geographic variation of least-square means of the traits based on the performance in the field trials: a) height at 9 months at Dungrove (cm) (ht_july11), b) height at 36 months at Dungrove (cm) (ht_nov13), c) height at 7 months at Meadowbank (cm) (ht1_may12), d) height at 20 months at Meadowbank (cm) (ht3_june13), e) proportion of plants with tips damaged by the insect *Perperus malevolens* at 18 months at Dungrove (tipdam_apr12), and f) proportion of plants showing drought damage at 15 months at Meadowbank (drought_jan13).

amongst populations (brows_nov12; $P > 0.05$). However, after the drought, when the trial had been heavily browsed by sheep, significant variation among populations was evident (brows_jun13; $P < 0.001$). In addition to population differences in susceptibility to biotic stresses such as browsing, populations also exhibited significant differences in their susceptibility to climatic stress from drought (Meadowbank drought_jan13; $P < 0.01$) and frost (Dungrove frost_apr11, $P < 0.001$).

As with the Tasmanian populations, the mainland populations differed significantly for height at all assessments (Table 5.3). However in contrast to the Tasmanian populations, there were significant differences in most survival assessments. As with Tasmanian populations, earlier insect damage and deer damage were significantly different among populations but not the later assessments. In contrast to the Tasmanian populations, there were no significant differences among the mainland populations for the later browsing at Meadowbank (brows_jun13) and drought damage (drought_jan13). However the mainland populations did differ in their levels of frost damage at Dungrove (frost_apr11; $P < 0.001$).

5.3.3 Association of traits with mating system parameters and climatic variables

Analysis of the effect of mating system parameters on seedling performance in the field trials provided evidence of significant inbreeding depression on growth (height) at the family level (Table 5.4). Inbreeding depression for height growth increased with age at Dungrove from 26% to 37%. Outcrossing rate differences between populations did not explain the significant variation among populations in height, except 9 months after planting at Dungrove (ht_july11). However, the trend was the reverse to what was expected with inbreeding depression, populations with higher outcrossing rates tended to be shorter. When significant, this trend only explained a small percentage of the variation (ht_july11 R^2 = 7%), and subsequent growth measurements were not significantly related to outcrossing rate. With the exception of height as assessed one month after planting (family t_m for ht0_nov10 R^2 28%, $P = 0.017$), no significant effects of outcrossing rate on growth or survival were observed at the family or population level at Meadowbank. In contrast to outcrossing rate, population variation in biparental inbreeding did appear to have a slight negative effect on population growth consistent with inbreeding depression. However while this effect appeared to be increasing with

age at Dungrove, it was only statistically significant ($P < 0.05$) at Meadowbank and was only evident at two ages (height_may12 and height_nov12). There was no significant effect of biparental inbreeding on population survival at either site.

Table 5.4. The relationship of growth and survival traits with outcrossing rate (t_m) and inbreeding depression due to selfing (%ID) at the family level, and population-level relationships of these traits with outcrossing rate (t_m) and biparental inbreeding (t_m-t_s) in *Eucalyptus pauciflora*. The population level associations were analysed using the regression of the population least-square means on the population-level estimates of outcrossing rate and biparental inbreeding presented in Chapter 2. Trait codes are detailed in Table 5.2.

Traits	Family t_m fitted as covariate in individual tree mixed model				Population level regression					
					t_m			t_m-t_s		
	Intercept	Slope	Prob	% ID	R^2	Slope	Prob	R^2	Slope	Prob
Dungrove trial site										
ht_nov10	15.2	5.44	0.009	26.4	4%	-7.24	0.090	0%	0.78	0.883
ht_july11	35.7	8.00	0.046	18.3	7%	-17.44	0.019	2%	-11.94	0.195
ht_may12	62.2	20.52	0.004	24.8	2%	-21.20	0.184	2%	-22.25	0.254
ht_nov12	69.2	15.46	0.055	18.3	4%	-26.34	0.094	3%	-26.85	0.163
ht_may13	76.0	38.85	0.001	33.8	1%	-21.14	0.320	4%	-45.18	0.080
ht_nov13	72.2	41.73	0.001	36.6	0%	-6.69	0.757	5%	-47.44	0.069
survival_may13	0.79	0.09	0.200	10.5	10%	0.26	0.052	0%	-0.001	0.998
Meadowbank trial site										
ht0_nov11	16.7	6.53	0.017	28.1	3%	-7.74	0.274	8%	-14.24	0.096
ht1_may12	32.3	3.59	0.351	10.0	1%	-4.20	0.607	14%	-21.53	0.025
ht2_nov12	45.4	6.41	0.259	12.4	1%	-7.76	0.643	14%	-44.22	0.025
ht3_jun13	35.1	10.81	0.140	23.5	0%	-3.99	0.838	9%	-42.54	0.069
survival_jun13	0.7	0.10	0.194	12.1	3%	-0.13	0.286	0%	0.04	0.803

Of the traits that showed significant differences among the Tasmanian populations in the field trials, the population least-square means for approximately 50% were significantly associated with altitude and/or climatic variables predicted for the site of population origin (Table 5.5). This climate association was particularly strong with the mean maximum temperature of the warmest month (TMXWM), which was the climatic variable best explaining the variation in many of the seedling traits (Chapter 4). At Dungrove, populations originating from lower altitude and warmer sites initially were taller, but this trend diminished with age to become non-significant by 24 months (Table 5.5). This trend was also evident for the mainland populations at both Dungrove and Meadowbank, except the positive relationship with TMXWM persisted longer.

There was no association of population variation in survival with altitude or any climatic factor for either the Tasmanian or mainland populations.

Biotic damage to the Tasmanian populations was significantly associated with population altitude or TMXWM climate for five of the six significant damage traits (Table 5.5). In these five cases, browsing or other biotic damage was greater in populations originating from lower altitude and warmer sites. The same trend was evident for the mainland populations but only statistically significant in four of the ten regressions. There was no evidence for an association of drought damage at Meadowbank with population altitude. However, drought damage was significantly negatively correlated with the mean maximum temperature of the warmest month (TMXWM; R^2 = 15%, $P < 0.05$) and the best climatic variable was the mean moisture index of the highest quarter (MIMHQ) which explained 48% of the variation amongst the Tasmanian populations. The same trend was evident but not significant in the mainland populations. The differences observed amongst the Tasmanian populations for frost damage were not associated with altitude nor any climatic variable. However for the mainland populations there was a trend for lower altitude population to be more damaged by frost (frost_apr11 R^2 = 32%, $P < 0.05$).

Multiple regression analyses rarely resulted in a statistically better prediction of the variation in population least-square means than the best climatic variable alone. There was only one exception where mean annual temperature (TANN) and coefficient of variation of rainfall (RCVAR) together explained 66% of the variation in population differentiation in browsing damage (brows_nov13). In no case did a mating system parameter (t_m or t_m-t_s) have better predictive power than altitude, TMXWM or the best climatic variable, and in no case when they were included with the climatic variables in multiple regressions did these parameters contributed significantly to the model.

Table 5.5. Association of growth, survival and damage traits with altitude, mean maximum temperature of warmest month (TMXWM) and other climatic variables of the site of origin of the Tasmanian and mainland *Eucalyptus pauciflora* populations. Only traits for which significant differences were detected amongst the Tasmanian populations are shown (see Table 3), except for pre-drought browsing at Meadowbank (brows_nov12). Trait codes are detailed in Table 5.2. For the Tasmanan populations, the BIOCLIM climatic variable which had the highest regression R^2 and the directionality of the relationship are shown.

Trait	Mainland						Tasmania						
	Altitude			TMXWM			Altitude			TMXWM			
	R^2	Slope	Sig	R^2	Slope	Sig	R^2	Slope	Sig	R^2	Slope	Sig	Best climatic factor
Dungrove trial site													
ht_nov10	54%	negative	**	50%	positive	***	53%	negative	***	46%	positive	***	TWMQ(+)52%
ht_july11	31%	negative	*	63%	positive	***	36%	negative	***	36%	positive	***	TIT(+)45%
ht_may12	2%	negative	ns	31%	positive	**	18%	negative	**	23%	positive	***	ns
ht_nov12	0%	negative	ns	21%	positive	*	8%	negative	ns	9%	positive	ns	ns
ht_may13	0%	positive	ns	19%	positive	*	1%	negative	ns	0%	negative	ns	ns
ht_nov13	0%	positive	ns	15%	positive	*	3%	positive	ns	1%	negative	ns	ns
survival_may13	25%	positive	ns	6%	negative	ns	1%	positive	ns	0%	positive	ns	ns
ins_nov10	16%	negative	ns	7%	positive	ns	41%	negative	***	34%	positive	***	TWMQ(+)39%
tipdam_apr12	5%	positive	ns	7%	negative	ns	8%	positive	ns	4%	negative	ns	ns
frost_apr11	32%	negative	*	15%	positive	*	6%	negative	ns	1%	positive	ns	ns
deer_nov12	19%	negative	ns	28%	positive	*	40%	negative	***	51%	positive	***	TMXWM(+)51%
brows_nov13	28%	negative	*	6%	positive	ns	60%	negative	***	50%	positive	***	TANN(+)60% ^
Meadowbank trial site													
ht0_nov11	60%	negative	***	31%	positive	**	1%	negative	ns	0%	negative	ns	ns
ht1_may12	13%	negative	*	9%	positive	ns	4%	positive	ns	5%	negative	ns	ns
ht2_nov12	7%	positive	ns	0%	positive	ns	4%	positive	ns	5%	negative	ns	ns
ht3_Jun13	3%	positive	ns	2%	negative	ns	8%	positive	ns	14%	negative	*	ns
drought_jan13	12%	positive	ns	7%	negative	ns	10%	positive	ns	15%	negative	*	MIMHQ(+)48%
brows_nov12	25%	negative	ns	23%	positive	ns	25%	negative	**	17%	positive	**	MIANN(-)41%
brows_Jun13	33%	negative	*	3%	positive	ns	16%	negative	*	19%	positive	**	MIMHQ(-)37%

TWMXW = mean maximum temperature of the warmest month, TWMQ = mean temperature of warmest quarter, TIT=isothermality, TANN = mean annual temperature, MIMHQ = mean moisture index of highest quarter, MIANN = mean annual moisture index, RCVAR = coefficient of variation of rainfall. Significance levels are: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns $P \geq 0.05$; (+) denotes the positive association; (-) denotes the negative association; ^ significant multiple regression with TANN(+) RCVAR(-)66%.

5.3.4 Genetic variation within Tasmanian populations

Significant within population genetic variation was observed only in 8 of the 29 performance traits measured across both trials, as evidenced by significant family within population variance (Table 5.3). Height measurements and insect damage were initially significantly different among families within populations, but the differences decreased and were not significant at later ages. Despite significant population differences in drought damage (drought_jan13) at Meadowbank, there was no evidence of significant variation between families within population. In contrast, for frosts susceptibility (frost_apr12) there was evidence of significant differences among families within populations as well as among populations.

5.3.5 Local versus non-local populations

For the Tasmanian populations, the significance of the pair-wise tests of populations against the closest local population to each of the trials sites is shown for key performance traits on the plots in Fig. 5.5. To further explore the performance of local versus non-local populations, the distance of 27 km was considered as an operational limit for the definition of a broader 'local' population, taking into account the results from the molecular markers (Chapter 3) and seedling morphology (Chapter 4) studies. Based on this criterion, at Dungrove [25] five other populations [17, 20, 29, 30 and 31] and at Meadowbank [Curringa, 35] three other populations [29, 30 and 34] could be defined as local populations. At Dungrove at 9 months of age, six populations [15, 19, 27, 28, 32 and 33] out of the 31 so defined non-local populations (originating from lower altitude and warmer sites) outperformed the closest local population [25] in height (ht_july11), while two populations [13,14] performed significantly worse than the closest local population (Fig. 5.5, significant populations were represented by asterix). None of the broader defined local populations performed significantly worse or better than the Dungrove [25] population at this age. However the trend changed by 36 months (ht_nov13), with no local or non-local population performing significantly better than the onsite Dungrove population, but four non-local populations [6, 9, 14 and 36] and one local population [31] performing significantly worse than the Dungrove population. At Meadowbank, Curringa [35] was the nearest population to the site and while not 'onsite' was considered as the control population for pair-wise comparisons. Initially, at seven months after planting (ht1_may12) 12 populations [2, 3, 7, 10, 15, 16,

18, 23, 26, 27, 28 and 33] out of the 33 non-local populations (with reference to [35]) significantly outperformed the Curringa population and one [29] of four local populations performed significantly better. Later at 20 months after planting (ht_jun13), only seven non-local populations [3, 7, 10, 16, 23, 26 and 27] and one local population [29] performed significantly better than the Curringa population. No local and only one non-local population [5] performed significantly worse than the Curringa populations at the Meadowbank.

5.4 Discussion

5.4.1 Population differentiation and association with altitude and temperature of origin

There was highly significant variation in field growth (as measured by plant height) among Tasmanian populations and the variation was evident throughout the assessments until the age of 3 years and in both trials. Initially, there was a family effect on height growth. But its disappearance in later stages suggests the initial large difference in height might reflect the nursery effect where seedlings were grown in family blocks before being randomized. Though variation in survival was not evident among population, there was significant variation on seven out of 10 fitness related traits. Early population variation observed in growth and most of the damage traits in the field trials provided evidence of broad-scale climate adaptation. This adaptive variation appeared to be in response to two independent facets of climate variation. First was the multi-trait response to temperature variation associated with altitude. The second appeared to be an adaptive response to low moisture availability as expressed in the response to drought.

Initially (up to 19 months after planting) populations originating from lower altitude, warmer regions appeared to outperform the local populations at Dungrove in early seedling height growth. At the same time populations originating from lower altitude and warmer regions appeared to be more susceptible to the insect damage and herbivore. The change in population differences in early performance appeared to reflect a trade-off between fast growth and herbivore susceptibility, which in the presence of herbivores, appears to result in the eventual loss of any association between growth and climate with age. Populations at warmer lower altitude sites appear to be adapted for fast early seedling growth (present chapter), with high resource allocation into growth

and structural storage tissue (e.g. lignotuber development – Chapter 4) but reduced allocation of resources into direct herbivore defences (e.g. stem oil glands – Chapter 4). Significant, genetic-based differences in seedling growth and morphology amongst populations from different altitudes was previously recorded in mainland *E. pauciflora* by Pryor (1956). As in our study, Pryor (1956) observed the trend of increasing tree height with decreasing altitude, the correlation being close to linear. Genetic-based adaptation of the photosynthetic physiology to altitude has also been reported for mainland *E. pauciflora* populations (Slatyer 1977a; Slatyer and Ferrar 1977a; Slatyer and Morrow 1977) with lower optimal temperatures for photosynthesis (Slatyer and Ferrar 1977a) at higher altitudes. Similarly, experiments using mainland populations of *E. pauciflora* from different altitudes (33 m to 1790 m) have shown the low altitude populations grow faster than high altitude populations and the growth response is more tolerant to high temperatures (Paton 1980).

Insect damage and herbivore susceptibility of populations was highly correlated with altitude and the temperature at the site of origin. Populations originating from lower altitude and warmer regions were more susceptible to the insect damage and herbivore. The reason for less susceptibility to hervivore of populations at higher altitudes could be due to adaptation to higher pressure exerted by hervivores, such as insects, and the evolution of resistance towards them (direct effect). Another explanation could be as lower altitude populations are growing faster, they might have more young leaves, and younger leaves are more favoured by the insect hervivores (Lowman and Box 1983). This altitudinal trend could also be due to an indirect effect associated with a correlated response to selection on other functional traits. However, this is not consistent with at least the one putative defensive trait studied in the glasshouse trial (Stem oil glands), which was genetically uncorrelated with all other seedling traits studied (Chapter 4). A study of the eucalypt leaf beetle (*Paropsisterna bimaculata*) and introduced *E. nitens* in Tasmania (Wardlaw *et al.* 2011), reports that both leaf beetle populations and their damage was greater at high altitudes and the severity was even more within the 10 km distance of *Poa* grassland. As the *E. nitens* is recently introduced for plantation purposes and most plantations are in their first rotation, genetic-based adaptation to the insect pressure is not expected to be confounded with this response. The report of Wardlaw *et al.* (2011) is consistent with greater herbivore pressure in high-altitude

woodland sites where *E. pauciflora* grows. Being a native species, *E. pauciflora* may have been able to counter herbivore pressure by genetic adaptation, hence resulting in the higher altitude population being more resistant to insect and other herbivore damage. Greater insect herbivory at higher altitudes in Tasmania has also been reported in *E. gunnii* (Potts 1985). However, in native *E. pauciflora* forest on mainland Australia the opposite trend was observed (Burdon and Chilvers 1974), but unlike the *E. nitens* example, this trend may be confounded with genetic-adaptation of the *E. pauciflora* itself reducing herbivory in the high altitude population. The differences between studies might also reflect differences in the broad chemical and physical traits affecting herbivory as well as different types of herbivore, time of assessment and the way the damage is assessed (Andrew *et al.* 2012).

In the glasshouse trial reported in Chapter 4, there was evidence of morphological adaptation of *E. pauciflora* populations to the climate and altitude of origin. Population variation in 13 out of 25 traits studied showed a significant association with altitude, and the best predictor for 7 traits was the mean maximum temperature of the warmest month. The field trials using the same populations as in the glasshouse trial also showed a similar trend. Around 50% of the field measurements that showed significant differences among populations were explained by the same climatic variable, the mean maximum temperature of the warmest month, and most of these also had a significant correlation with altitude of population origin. The adaptive nature of this altitudinal variation in early growth and some of the browsing damage traits is supported by parallel trends being detected in both mainland and Tasmanian populations (Table 5.5). However, at Dungrove the differences in browsing damage amongst populations were more related to temperature whereas at Meadowbank they were more related to the moisture indices of the site of origin. This may reflect differences in foliar quality associated with the drought damage, but could also reflect a change in browsing preferences associated with a stress response. For example, changes in biotic stress to eucalypt trees following drought damage have been previously reported (Caldeira *et al.* 2002; Hanks *et al.* 1999). In general Meadowbank has higher mean maximum temperature and receive more precipitation than Dungrove, but over the past two years, due to recorded high temperature heat stress and the recorded low precipitation (Bureau of Meteorology 2013) appear to have impacted the Meadowbank site more, resulting in

the performance differences in two trials. While this may have been due to different climates being experienced at the two sites during the establishment period, localised site characteristics and the fact that the Dungrove site had an addition year of establishment prior to these events cannot be dismissed as factors explaining the absence of noticeable drought damage at the Dungrove site at the same time as Meadowbank.

Adaptation to altitude is well documented in many eucalypt species (Potts and Wiltshire 1997; Potts and Jackson 1986). Many other species also show strong altitudinal and localized temperature adaptation in physiological and growth traits (Grady *et al.* 2011; Oleksyn *et al.* 1998; Rweyongeza *et al.* 2007; Vitasse *et al.* 2009). Though in our study, Tasmanian populations did not provide any evidence that frost damage was related to climate or altitude, populations from the mainland did show decreased frost damage with increase in altitude of origin. A broad-scale genetic-based adaptation for increasing frost resistance with increasing altitude of origin has been demonstrated in several studies of mainland *Eucalyptus pauciflora* (Green 1969b; Paton 1980; Pryor 1956). Subsequent studies also revealed a complex pattern of altitudinal differences in frost resistance which may occur over short-distances, involving strong selection near the upper and lower tree lines (Harwood 1980; Harwood 1981). Such fine-scale adaptation to frost could explain the absence of a broad-scale altitudinal and climatic response observed for the Tasmanian populations.

Significant genetic differences in drought damage among the Tasmanian populations appeared to reflect an adaptive response to low moisture availability. While no association was observed with altitude of origin, our study showed populations originating from sites with lower mean moisture index of the highest quarter (MIMHQ) and higher mean maximum temperature of the warmest month (TMXWM) were less susceptible to drought damage. Also the present study suggests that of these two climatic variables, population differences in response to the specific drought/heat stress event that impacted the Meadowbank trial is more related to adaptation to water stress than to heat stress. Nevertheless *E. pauciflora* is known to have the ability to withstand periodic drought and a combination of environmental stresses (Williams and Ladiges 1985; Williams and Potts 1996). This was certainly the case for the damage that

occurred at Meadowbank which was related to a record extreme hot temperatures in Tasmania, combined with very dry conditions (Bureau of Meteorology 2013). Adaptive divergence in drought stress has been recorded in *E. globulus* (Costa e Silva *et al.* 2006; Dutkowski and Potts 2012) and similar to our studies, Dutkowski and Potts (2012) observed that Tasmanian populations originating from sites with higher water availability and low evaporation were more susceptible to drought stress.

5.4.2 Population differentiation is not affected by variation in outcrossing rate

Population variation assessed using open-pollinated progeny may be confounded by differences in inbreeding, particularly in growth traits (Potts and Wiltshire 1997; Potts and Jordan 1994). Inbreeding depression has been associated with reduced growth and survival in many *Eucalyptus* species (Eldridge and Griffin 1983; Potts and Wiltshire 1997; Potts and Jordan 1994; Potts *et al.* 1987) and with age inbreeding depression can eventually result in nearly complete elimination of selfed individuals (Costa e Silva *et al.* 2010; Griffin and Cotterill 1988). In the present study, analysis of the effect of mating system parameters (outcrossing rate and biparental inbreeding) on the performance of the seedling in the field trials provided evidence of significant inbreeding depression due to selfing on height growth at the individual tree level ranging from 18 to 33% (Table 5.4). A similar trend of increasing inbreeding depression in height was observed by Hardner and Potts (1995) in selfed *E. globulus* (from 17 % at 10 months to 26 % at 43 months). The level of inbreeding depression observed for height in *E. pauciflora* is slightly higher than the levels reported from selfing other eucalypts species (Griffin and Cotterill 1988; Hardner and Potts 1995; Hardner and Tibbits 1998), and this could be related to the relatively high outcrossing rate in this species (Chapter 2) leading to the accumulation of more deleterious recessive genes in the large populations.

At the population level, there was no trend for growth to be related to levels of inbreeding. Neither were there significant effects of population differences in outcrossing rate on survival rates in either trial. The effect of variation in biparental inbreeding at the population level was never significant at Dungrove, while it did adversely affect intermediate height growth at Meadowbank. *Eucalyptus pauciflora* has high outcrossing rate with only 10 % selfing (Gauli *et al.* 2014), which might be the reason for the minimal effect of inbreeding depression on the fitness traits at population

level. Overall, the results suggest that while inbreeding depression at family level is significant in this species, this has a minimal impact on variation between population which is likely more affected by additive genetic variation between populations. As the present study was only based on early growth and survival until the age of 3 years, the effect of inbreeding at the population level and on survival at the individual level may become more evident with age.

5.4.3 Local adaptation to the experimental sites

5.4.3.1 Translocation of mainland genotypes to the island of Tasmania

Movement of seeds or seedlings is frequently undertaken to restore the landscape and this has given rise to considerable debate about the optimal seed sourcing strategy for restoration while conserving the existing genetic variation patterns within species (Broadhurst *et al.* 2008; Krauss *et al.* 2007; McKay *et al.* 2005). Though local genotypes are often considered to be best adapted (Hufford and Mazer 2003; Kramer and Havens 2009; O'Brien *et al.* 2007), in the face of climate change, several studies have raised concerns over the suitability of local genotypes for the habitat of the future (Byrne *et al.* 2011; O'Brien and Krauss 2010; Sgrò *et al.* 2011). Integration of mainland genotypes in the restoration of the Tasmanian midlands showed, from the initial stage, that the Tasmanian population clearly outperformed the mainland populations. In addition to height growth, survival rate was also greatly different between Tasmanian and mainland populations with higher survival rate for the Tasmanian populations. Despite insect and deer favouring fast growing the Tasmanian populations, they were performing far better than the mainland populations hence arguing against movement of germplasm from mainland to Tasmania for better restoration outcomes.

5.4.3.2 Local vs non local issues within Tasmania

Within Tasmania, at the more benign, mid-altitude site at Dungrove, there was little evidence of provenance choice impacting early growth or survival, despite earlier (until 19 months) indications that populations from warmer regions were favoured. At the final measurements, though there were populations from warmer lower altitude region growing better, they were not significantly better than from the immediate site and where significantly poor performance was detected there was just as much likelihood of a ‘local’ (within 27 km) population performing poorly as a non-local population. By

contrast at Meadowbank, there was a clear signal that non-local populations were favoured over the closest local population (in terms of less drought damage and last height measurement). While adaptive differences in growth and fitness traits may be evident in the early age (Bush *et al.* 2013; Dutkowski and Potts 2012), sometimes it may also take several years until it is evident (Chambers *et al.* 1996; Lopez *et al.* 2003). Sudden pulses of harsh environmental conditions such as frost or drought may reshape the adaptation dynamics of fitness traits of the populations (Kay and Picklum 2013; Montalvo *et al.* 1997). This may result in trade-offs between traits (Petit and Hampe 2006; Sgrò and Hoffmann 2004) as a differential response to selection. At Meadowbank, the occurrence of the combined stress of drought and a high temperature weather event (potentially coupled with the effects of a surrounding wildfire), differentially affected populations at the establishment phase and may change the long-term growth dynamics and selective outcome on the site. In addition, the changing growth dynamics at Dungrove due to what appears to be a trade-off between rapid growth and defence against herbivory, suggests that while adaptive patterns in early growth can be revealed, the plants have just established and the clear genetic differences in other traits such as herbivory and drought susceptibility may change the selective outcome with time.

In conclusion, there are significant population differences in *E. pauciflora* for growth, survival and the susceptibility to biotic and abiotic stresses. While there is evidence that the differences observed among populations appears to reflect historical adaptation to altitude and climate of origin, there was no clear evidence that local genotype in the broad- (<27 km) and narrow (closest site) sense are better adapted than non local genotypes at this early establishment phase. However, the occurrence of ongoing (e.g. herbivory) and more-catastrophic selective events (drought), emphasized the dynamic nature of seedling growth and suggests that with more time it is possible that local populations may start performing better. Nevertheless our result clearly indicated that Tasmanian populations outperformed the mainland populations in this establishment phase, and coupled with the diversity present within Tasmanian *E. pauciflora* argues against the need for seed translocation from mainland at least in the current climate. The recommendation on selecting and sourcing the best Tasmanian provenance for restoration purpose from this early age data remains difficult due to the dynamic nature of this early establishment phase and different responses evident in the two trials.

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Chapter 6. Discussion and conclusion

This thesis has advanced our knowledge on the mating system, genetic diversity and adaptation in *Eucalyptus pauciflora*. The major findings of the study are overviewed below in terms of the original issues raised and relevance of findings to local adaptation and choice of seed sources for use in restoration.

6.1 Minimal impact of habitat fragmentation on genetic variation in fitness traits in *Eucalyptus pauciflora*

By investigating mating system parameters of *E. pauciflora* populations exhibiting diverse levels of forest fragmentation, it is now apparent that although forest fragmentation appears to reduce early seed yield, there is a little effect on the mating system parameters of *E. pauciflora*. Decreased population size due to habitat fragmentation is often considered to disrupt the gene flow dynamics of the population (Vranckx *et al.* 2012). Disruption in gene flow mechanism can lead to increased selfing and, thus, increase inbreeding depression, which can have strong fitness impacts on progenies (Lowe *et al.* 2005; Young *et al.* 1996). Though the impact of fragmentation can be deleterious, species can prevent this through the utilization of self – incompatibility mechanism (Husband and Schemske 1996). The minimal effect of fragmentation on mating system parameters we observed could be due to late acting post–zygotic incomplete self-incompatibility mechanisms, which are common among the eucalypts (Horsley and Johnson 2007; Pound *et al.* 2002). This may have buffered *E. pauciflora* against the expected negative consequences of forest fragmentation (Byrne *et al.* 2008; Kramer *et al.* 2008). Populations of *E. pauciflora* do vary in their outcrossing rate, but there is no evidence to accept the original hypothesis that forest fragmentation contributes to this variation. While it has been cautioned that seed collected from fragmented populations may exhibit increased rates of inbreeding, and harbour reduced genetic diversity, the present study did not support this hypothesis. In most cases the open-pollinated seed collected from fragmented *E. pauciflora* populations is unlikely to be more inbred or less genetically diverse than that from non-fragmented populations. Thus while germinant yields per capsule collected may be reduced in this species because of fragmentation, the seed will be suitable for use in

local forest restoration. It is worth noting that the low seed yield of fragmented trees means higher cost for seed collection.

6.2 Molecular markers provide insights into past migration and contemporary gene flow

By exploring the genetic pattern of chloroplast and nuclear diversity in *E. pauciflora* populations, this thesis enabled a new understanding of the historical processes and patterns of gene flow which have shaped the *E. pauciflora* gene-pool on the island of Tasmania. In particular the molecular information has provided insights into the species response to past climate changes, including its distribution during glacial periods and migration patterns following post-glacial range shifts. The distribution of chloroplast haplotype richness showed a clear geographic pattern, with low-altitude centers of high richness suggestive of three major glacial refugia. Low haplotype richness and population sharing of haplotypes suggest post-glacial colonisation of the Tasmanian highlands, probably from lowland populations rather than *in situ* high-altitude source populations. Higher nuclear genetic diversity in putatively newly colonized areas compared to lowland putative refugial regions, and the converse in chloroplast DNA markers, suggested limited seed dispersal into newly colonised regions combined with high pollen flow between different source populations in newly colonised areas, since chloroplast are only seed transmitted while nuclear microsatellites are transmitted by both seed and pollen (Brondani *et al.* 2006; McKinnon *et al.* 2004a). This study has contributed to the increasing understanding of the effects that the Pleistocene glacial cycles have had on shaping the contemporary eucalypt gene pools and species distributions on the island of Tasmania.

In addition, the molecular study revealed insights into the patterns of gene flow in *E. pauciflora*. The highly spatially structured (high F_{ST} values) genepool of *E. pauciflora* in maternally inherited, chloroplast markers compared with the low F_{ST} between populations for the nuclear markers argue that gene flow is mainly pollen-mediated, consistent with most eucalypts studied to date (Byrne 2008b). The higher levels of heterozygosity and lower F_{ST} values detected in mature forest maternal samples compared with the progeny samples, is consistent with selection against the products of inbreeding which are more likely associated with proximal (bi-parental) matings. This

hypothesis is consistent with the evidence for early-age inbreeding depression that was revealed by the two common garden field trials. However, variation in mating system parameters, particularly outcrossing rate, did not explain population variation in early age fitness-related traits. Rather, many of the functional traits studied exhibited much greater population-level inbreeding (high Q_{ST}) than the nuclear markers would have predicted. These quantitative and molecular comparisons argue that disruptive selection rather than drift has shaped the patterns of genetic variation in many traits and over-ridden the historical signals evident in the chloroplast haplotypes and more recent pollen-mediated gene flow.

6.3 Altitude and temperature of warmest month - key drivers of population divergence

Analysis of quantitative traits variation in seedling morphology in *E. pauciflora* provided strong evidence of adaptation to climatic factors of the site of origin. This adaptive genetic variation was associated with altitude, and particularly with the mean maximum temperature of the warmest month which increases with decreasing altitude. Apart from that expected from pollen-mediated gene-flow, there was no evidence of an association of geographic distance and the quantitative genetic variation among populations. With most of the traits association with climate variables, it is argued that small changes in climate, such as a 1ºC change in the maximum temperature of the warmest month, are likely to lead to mal-adaptation of local populations of the species. However, in addition to the potential for the redistribution of genetic variation amongst populations through pollen-mediated gene flow, there was evidence for significant levels of additive genetic variation residing within populations for most of these key functional traits which will allow a response to selection. This study provided evidence that adaptation of *E. pauciflora* to climate change may involve parallel changes in multiple plant traits. The analysis of intra- and inter-population genetic correlations argues that the parallel response patterns exhibited by multiple traits to changes in the same climatic gradient is controlled by different genes, rather than reflecting a correlated responses to selection arising from pleiotropy or linkage. This finding highlights the evolutionary significance of individual seedling traits, including those associated with resource allocation (lignotuber development), ontogenetic development (transition to an alternate leaf type) and biotic defence (stem oil glands).

Population variation observed in early-age performance traits in the field trials also provided evidence of broad-scale climate adaptation in the Tasmanian *E. pauciflora*. The adaptive genetic variation revealed in the field trials appeared to be in response to two independent facets of climate variation that occurred across the range of *E. pauciflora*. First was the multi-trait response associated with altitude which was mainly driven by temperature variation. Height growth and susceptibility to insect herbivores and browsing damage showed this trend, though the association for height decreased with age and was eventually erased. The genetic adaptation of drought damage was however associated with the low moisture availability.

6.4 Delineation of local seed source

This thesis provides several lines of evidence to define a spatial scale for an operational local population in *E. pauciflora*. Spatial structuring of genetic variation is a consequence of evolutionary drivers such as phylogeographic history, local adaptation and restricted gene flow, and therefore the key consideration for defining the local provenance (Krauss *et al.* 2013). In our study, both nuclear microsatellite (putatively neutral loci) analysis on maternal and progenies samples and morphological traits analysis (traits of adaptive significance measured at the seedling and young tree phase) showed that there is an above average genetic similarity of populations within the distance of 27 km. As the molecular comparisons of chloroplast and nuclear markers suggest that gene flow is predominantly pollen-mediated in *E. pauciflora*, the significant nuclear genetic similarity over these distances is most likely indicative of the distance over which broad-scale pollen dispersal is likely to define the local population. The suggestion of 27 km as an operational limit for defining a local population is congruent with the recommendation of the threshold local population seed collection zone of 30 km (radius) for *Banksia menziesii* for ecological restoration (Krauss *et al.* 2013). In contrast, the Western Australian Forest Management Plan 2004-2014 advocates the distance of 15 km for seed collection (Broadhurst *et al.* 2008), which would be more conservative than our recommendation for *E. pauciflora*, but consistent with the observed tree-level increase in genetic similarity amongst trees separated by 10 km.

6.5 Is there direct evidence that local populations are better adapted – implications for choosing a seed sourcing strategy for restoration

In the broad-sense the issue of what is local can be treated at the island-level, whereas the thesis compared the performance of Tasmanian *E. pauciflora* populations with that of introduced mainland populations in field trial. Over the next century, the climate envelopes of many eucalypt species are expected to exhibit a southward shift (Butt *et al.* 2013; Hughes *et al.* 1996), accordingly the translocation of mainland populations onto the southern island of Tasmania is likely to be an assisted migration strategy argued in the future. While the present Thesis only studied the establishment phase of tree plantings up to 3 years after planting, it was clear that on average the Tasmanian populations outperformed the mainland populations in terms of growth and survival in both field trials over this period. This response would argue against the need for translocation of mainland seed sources to Tasmania, at least under the current prevailing climate conditions. However, while current results signal mal-adaptation of mainland populations in Tasmania, insect herbivory and deer damage was greater on the faster growing Tasmanian populations which could change the relative fitness of survivors in the future, as could an increasingly warmer/dryer climate (Mok *et al.* 2012).

In the narrower sense, the issue of local provenance was addressed in terms of seed sourcing from within Tasmania and considering either the site-specific local population, or the set of populations within the identified 27 km operational limit of the local population. At the Dungrove planting site there was a little evidence of provenance choice impacting early growth or survival, despite early indications that populations from lower altitude warmer regions were favoured. At the final measurements, there was no population growing significantly better than the local population and where significantly poorer performance was detected there was just as much likelihood of another ‘local’ population performing poorly as a non-local population. By contrast at Meadowbank, there was a signal that non-local populations were favoured over the closest local population (in terms of less drought damage and last height measurement). However, only 1.5 years of data were available for Meadowbank and the possibility that changing growth dynamics as observed at Dungrove, due to what appears to be a trade-off between rapid growth and defence against herbivory, may eventuate at the Meadowbank and change the fitness profile in the future.

In conclusion, considering the long life cycle of the *E. pauciflora* and the diverse environments over which it grows it is obviously too early to suggest the suitability of local compared to non local populations. There are clearly a multitude of abiotic and biotic factors which impact on population fitness at different sites and at different times, including catastrophic events which this study shows may be site specific, including severe browsing and drought/heat stress. Making recommendations on seed sourcing strategy for restoration purpose in Tasmania from this early age data remains difficult due to the dynamic nature of this early establishment phase and different responses evident in the two trials. The differences in seedling performances in the two trials and the observed changing growth dynamics, thus make it difficult to either reject or accept the initial hypothesis that locally collected seeds sources from forest remnants might not always be better adapted than the seedlings raised from non-local seeds collected from intact native forest because of a combination of inbreeding and changing environments.

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