Acacia saligna as a Sustainable Agroforestry Crop for southern Australia: A Genetic Assessment

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Statement of Originality

This work contains no material that has been accepted for the award of any other degree or diploma in any other University or other tertiary institution. This thesis does contain material that has been either published or submitted for publication in peer-reviewed scientific journals.

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Abstract

Acacia saligna is a native species complex with a widespread natural distribution throughout the south west of Western Australia. It is being developed as an agroforestry crop to produce low value, bulk biomass products in the low rainfall agricultural areas of southern Australia. This thesis develops knowledge to assist the domestication and breeding program of *A. saligna* as an agroforestry cultivar. It also furthers development of a risk management plan for utilisation of the *Acacia saligna* species complex.

Highly informative microsatellite markers for *A. saligna* were developed for use in mating system studies, paternity analysis and in the development of a diagnostic tool for the identification of individuals and populations at the subspecific level. Microsatellites developed in other *Acacia* species were also screened for utility in *A. saligna*. A high level of outcrossing (mean multilocus outcrossing rate of 0.98) and little true selfing was found for a planted stand of *A. saligna* subspecies *saligna*. Paternity analysis indicated heterogeneity in pollen clouds experienced by maternal trees and an essentially random pattern of mating within the stand. Inter-subspecific pollen immigration into the stand from trees of subspecies *lindleyi* was detected for 14% of progeny analysed and occurred over distances greater than 1500 m. Extensive intra-subspecific pollen-mediated gene flow is maintained between remnant natural populations of *A. saligna* subspecies *lindleyi*, and a high level of inter-subspecific pollen immigration from trees in the planted stand of *A. saligna* subspecies *saligna* was detected in remnant populations of subspecies *lindleyi* (32% of analysed progeny) occurring over distances greater than 1500 m.

Polymorphic microsatellite markers used to investigate genetic structuring within *A. saligna* revealed a high level of genetic divergence between subspecific entities congruent with a taxonomic reclassification of the species complex. Selected microsatellite markers also proved suitable for use as a rapid diagnostic tool that can be used to characterise populations into one of the proposed subspecies of *A. saligna* with high probability.

These results indicate that high levels of outcrossing and essentially random patterns of mating that maintain genetic diversity in seed crops should be achievable with the suitable management of seed production stands of *A. saligna*. Appropriate management techniques that limit genetic contamination into seed production stands will need to be employed to achieve this goal. Management techniques will also be required to minimise the risk of genetic contamination from stands planted for agroforestry purposes into remnant natural populations. Isolation distances greater than 1500 m between genetically divergent agroforestry crops and natural populations are suggested in both cases and key areas of further research are suggested.

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Thesis by Publication

This doctoral thesis is submitted as a portfolio of publications either published or submitted for publication by peer-reviewed journals according to the 'PhD Rules and Specifications for Thesis' of the University of Adelaide. The journals in which these papers were published are closely related to the research field of this work. The citation information is listed in the order of their appearance in this thesis. The thesis is based on the following papers,

Millar MA, Byrne M, Nuberg I, Sedgley M (2008) High outcrossing and random pollen dispersal in a planted stand of *Acacia saligna* subsp. *saligna* revealed by paternity analysis using microsatellites. Tree Genetics and Genomes. 4: 367-377.

Millar MA, Byrne M, Nuberg I, Sedgley M (2008) Long distance inter-subspecific gene flow detected by paternity analysis in remnant *Acacia saligna* (Mimosaceae). Heredity. In prep.

Millar MA, Byrne M (2007) Characterisation of polymorphic microsatellite DNA markers for *Acacia saligna* (Labill.) H.L.Wendl. (Mimosaceae). Molecular Ecology Notes. 7: 1372-1374.

Millar MA, Byrne M, Nuberg I, Sedgley M (2008) A rapid PCR based diagnostic test for the identification of subspecies of *Acacia saligna*, Tree Genetics and Genomes. doi 10.1007/s1 1295-008-0138-0.

The following additional publications are of relevance to the present work and were published over the course of the doctoral candidature:

Millar MA, Byrne M, (2004) Genetic systems and issues in the development of woody perennials for revegetation. In: Ridley, A, Feikema, P, Bennet, S, Rogers, MJ, Wilkinson, R, Hirth, J (Eds) Proceedings of the Conference Salinity Solutions: Working with Science and Society, Bendigo Victoria CRC for Plant-Based Management of Dryland Salinity, 2-5 August, 2004: 1-5.

Millar MA, Byrne M, Nuberg I, Sedgley M (2005) Risk assessment for *Acacia saligna* agroforestry. Advances in plant conservation biology symposium; Implications for flora management and restoration, Perth, Western Australia, 25 - 27 October, 2005.

Millar MA, Byrne M, (2007) Pollen contamination in *Acacia saligna*: assessing the risks for sustainable agroforestry. In: Sixth International Conference on Ecosystems and Sustainable Development Tiezzi E, Marques JC, Brebbia CA, Jorgensen SE (Eds), Portugal, Coimbra, 3-6 September, 2007, 101-110.

Sampson JF, Millar MA, Byrne M (2007) Pollen dispersal between planted populations and remnant native populations in a fragmented agricultural landscape. In: Rokich D, Wardell-Johnson G, Yates C., Stevens J, Dixon K, McLellan R, Moss, G (Eds), The International Mediterranean Ecosystems Conference Perth, Western Australia, Kings Park and Botanic Garden, 2-5 September, 2007, 221-222.

Byrne M, Stone L, Millar M.A. (2007) Environmental risk in agroforestry, In: Agroforestry for Natural Resource Management, Nuberg I, George B, Reid R (Eds), CSIRO Publishing, in press.

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Account of Research Progress

This research has investigated a number of key areas to assist the domestication and breeding program and further the development of a risk management plan for utilisation of the *Acacia saligna* species complex as an agroforestry cultivar. The research is presented as a series of research papers written for submission to peer reviewed scientific journals. The progress of the research is described in the order of the chapters and papers presented in this thesis.

Chapter One

Chapter One is a general introduction to the thesis that introduces the context, study species and impetus for this research. The overall research aim is stated and the overall research objectives described.

Chapter Two

This chapter provides a background of the relevant literature underpinning this research. The need for the development of native woody perennial species as agroforestry crops is discussed in the context of increasing salinisation of the landscape and waterways of the agricultural areas of southern Australia. Relevant current knowledge regarding *Acacia saligna*, the priority species selected for further development as an agroforestry cultivar, is presented and areas where further knowledge of the species complex is required are identified and discussed. Suitable methodological approaches for investigating some of the key areas of research are introduced and the specific research objectives stated.

Chapter Three

This chapter presents a study of the mating system and patterns of intra-subspecific pollen-mediated gene flow within a planted stand of *A. saligna* subspecies *saligna*. The production of an initial unenriched microsatellite library and the development of two polymorphic microsatellite markers for *Acacia saligna*, and the screening of known microsatellite markers developed in other Acacia species is presented. Patterns of genetic contamination within the planted stand from inter-subspecific pollen immigration from natural remnant populations of subspecies *lindleyi* are assessed. The study is conducted using maximum likelihood paternity analysis of genotypic data at the five microsatellite loci.

Chapter Four

This chapter presents a study that investigates patterns of pollen-mediated gene flow. It investigates both intra-subspecific pollen-mediated gene flow among natural remnants of *A. saligna* subspecies *lindleyi* and patterns of inter-subspecific gene flow into natural remnants of subspecies *lindleyi* via pollen dispersal from a planted stand of subspecies *saligna*. The study is conducted using maximum likelihood paternity analysis of genotypic data obtained for the five microsatellite markers described in Chapter Three.

Chapter Five

This chapter details the production of a second microsatellite library using enrichment techniques, and the development of ten highly informative polymorphic microsatellite markers. A method incorporating several enrichment procedures was used to construct the microsatellite library because of the poor efficiency of primer development in the unenriched microsatellite library described in Chapter Three. The utility of the ten primers is described for two populations of *A. saligna*, one of subspecies *saligna* and one of subspecies *lindleyi*. These markers were screened for suitability of developing a diagnostic tool for the accurate identification of subspecies of *A. saligna*.

Chapter Six

Presents the development of a rapid and accurate diagnostic tool via Bayesian assignment analysis using genotypic data obtained for five of the diagnostic microsatellite markers described in Chapter Five. Its utility in characterising a number of cryptic populations of *A. saligna* at the subspecies level is presented.

Chapter Seven

This chapter is a general discussion of the how this work achieved the overall research objectives outlined, the main findings of the research and the principal significance of each of the main outcomes. A number of recommendations are made for the domestication and breeding program and for the sustainable management of *A. saligna* as an agroforestry cultivar. Key areas requiring further research for the development of *A. saligna* are identified and suitable approaches for further research are suggested.

Chapter One
General Introduction and Research Objectives

Introduction

Acacia saligna is a native species complex with a widespread natural distribution throughout the south west of Western Australia. The species complex shows high levels of ecological, morphological and genetic divergence and is informally described by four main subspecies. *Acacia saligna* has been selected by a number of studies as the priority native species for further development as a perennial agroforestry crop for the low rainfall agricultural areas of southern Australia (Bennell et al. 2004; Maslin and McDonald 2004; Olsen et al. 2004). Large-scale perennial plantings in the form of agroforestry crops are urgently required in the southern agricultural landscape to address increasing levels of salinisation in the land and waterways. It is envisaged that *A. saligna* be developed and used as a short rotation agroforestry crop for the production of bulk biomass, composite wood products and livestock fodder (Bartle et al. 2002; George 2005).

There are however a number of areas where further knowledge of the species complex would assist the efficient development and sustainable use of *A. saligna* as a large-scale agroforestry crop. In order to guide a domestication and breeding program and ensure the planting of *A. saligna* throughout its natural range does not negatively impact on remnant natural populations, it will be useful to investigate the mating system, and patterns of intra-subspecific and inter-subspecific pollen-mediated gene flow that may be expected in both planted and natural remnant populations of the species complex (Byrne and Millar 2004).

In order to achieve genetic gains in the breeding program and maintain genetic diversity within seed crops harvested from seed production stands such as seed orchards, it is desirable to maintain a high level of outcrossing, equal contributions of trees to seed crops and mating that approaches panmixia. It is also important to minimise genetic contamination via pollen-mediated gene flow from trees outside the production stand. Seed orchards do not yet exist for *A. saligna* and knowledge of the mating system and patterns of intra-subspecific and inter-subspecific pollen-mediated gene flow that can be

expected in planted stands of *A. saligna* will assist in the efficient design and placement of seed orchards for the production of seed of improved genetic value.

A similar approach will be of assistance in assessing the likelihood of inter-subspecific pollen immigration occurring in remnant natural populations in the south west of Western Australia. Inter-subspecific pollen immigration may negatively impact on the maintenance of genetic diversity in natural remnant populations and pose a threat to their long-term persistence. Extensive pollen immigration into remnants, which are often small and fragmented, may be expected if genetically divergent populations are planted for agroforestry on a large-scale across the species natural range. Assessment of the likelihood of inter-subspecific pollen-mediated gene flow will provide information of use in designing management guidelines that minimise the risk of pollen immigration in remnant populations.

Mating systems and patterns of both intra-subspecific and inter-subspecific pollen-mediated gene flow within and among populations can be studied directly using highly polymorphic DNA markers such as microsatellites (Ritland 2002; Nybom 2004). When maternal trees and a subset of their progeny are genotyped for a number of loci, maximum likelihood paternity analysis can be used to establish the most likely paternal tree for each progeny. This information can then be used to assess the mating system and, when combined with spatial analysis, to describe patterns of mating and the levels and distances of pollen-mediated gene flow.

Knowledge of the genetic entities and levels of genetic diversity between subspecific entities in *A. saligna* is a basic requirement for effective domestication and breeding, and is required to provide information on the likely impacts of hybridisation via pollen-mediated gene flow. The *Acacia saligna* species complex shows a considerable level of genetic structuring (George et al. 2006) although the genetic relationships between entities have not yet been fully resolved and further investigation is required. Genetic structuring can be assessed and population structure inferred within *A. saligna* using genotypic data such as that obtained with microsatellites and Bayesian model-based clustering methods (Manel et al. 2005) that do not rely on *a priori* information (Pritchard et al. 2000).

Due to the high levels of morphological and genetic diversity in the *A. saligna* species complex, difficulty is often experienced in identifying individuals and populations of *A. saligna* at the subspecies level in the field. A reliable PCR based diagnostic tool would provide a rapid and efficient means of accurately characterising individuals and populations at the subspecies level. Such a diagnostic tool would be valuable for seed collectors and breeders, to ensure the *in situ* and *ex situ* conservation goals for the species complex are achieved, in studies of invasive populations outside the species natural range, and for studies of hybridisation and introgression within the species complex. This tool could be developed using microsatellite genotypic data in conjunction with Bayesian clustering and assignment analysis (Manel et al. 2002; Falush et al. 2003; Manel et al. 2005).

Research Aim

The aim of this work was to provide information to assist the domestication and breeding program and in the development of a management plan for the environmentally sustainable use of the *Acacia saligna* species complex as an agroforestry crop for the low rainfall agricultural areas of southern Australia.

Research Objectives

The overall objectives of this work were to:

- Investigate the mating system, patterns of pollen dispersal and level and distance of inter-subspecific pollen immigration from natural populations that can be expected in seed production stands of *Acacia saligna*. The knowledge generated here informs the domestication and breeding program for the efficient management of seed production stands in which genetic gain is maximised and genetic diversity maintained in seed crops.

- Investigate patterns of genetic contamination via pollen flow from planted stands that can be expected in natural populations of *Acacia saligna*. With this knowledge, agroforestry populations of *Acacia saligna* can be managed to ensure that inter-subspecific pollen immigration into remnant populations from populations planted for agroforestry is minimised.

- Investigate genetic structuring present within *A. saligna* and assess the congruence with taxonomic reclassification of the species complex.

- Produce a diagnostic tool for the rapid and accurate identification of individuals and populations of *A. saligna* at the subspecies level.

Chapter Two
Literature review

The need to address increasing levels of salinisation in the Australian agricultural landscape necessitates the development of perennial native species as large-scale agroforestry crops. *Acacia saligna* has been selected as a priority species for further development due to it exhibiting a range of desirable attributes. Despite a long history of utilisation both in Australia and overseas, no advanced breeding material of *A. saligna* exists and a number of areas requiring further research have been identified for the development of the species complex as an environmentally sustainable agroforestry crop. This chapter introduces the issue of salinisation and sets the context for the development of native perennial species as agroforestry crops for southern Australia. It then introduces the current knowledge base regarding the *Acacia saligna* species complex and discusses a number of key areas of research that have been identified for the development of *A. saligna* and the methods by which this research could be addressed.

Vegetation Clearance and Salinisation

Vegetation Clearance

The biological integrity of Australian landscapes has been drastically reduced since European settlement. Loss of biodiversity is widely recognised as Australia's most important environmental problem (DEST 1996) and 'first and foremost, by far the major factor negatively affecting Australian biodiversity is the clearance of natural vegetation' (Working Groups on Biodiversity 1992). Since European settlement, an estimated 13% of Australia's native vegetation has been cleared (Figure 2.1), the majority of this being native forests and woodlands of southern Australia (Glanznig 1995). Removal of native vegetation in areas such as coastal regions of eastern Australia and the Western Australian wheat and sheep belt has been almost complete (Floyd 1987; ACF 2001). The majority of this clearance has been for agricultural expansion and production in what is now Australia's intensive agricultural zone (Barson et al. 2000).

The impacts of extensive vegetation clearance in Australia's agricultural areas are severe and ongoing: direct losses to the compositional biodiversity of terrestrial, riparian and aquatic communities, and a general decline in the health of functional

biodiversity that provides essential ecosystem services (Cogger 1991; Bennett 1993; Hatton et al. 2003). The disruption to essential ecosystem services is clearly seen in the salinisation of landscapes and freshwater sources, a result of alterations to the natural hydrological balance brought about by the removal of deep-rooted perennials from the landscape.

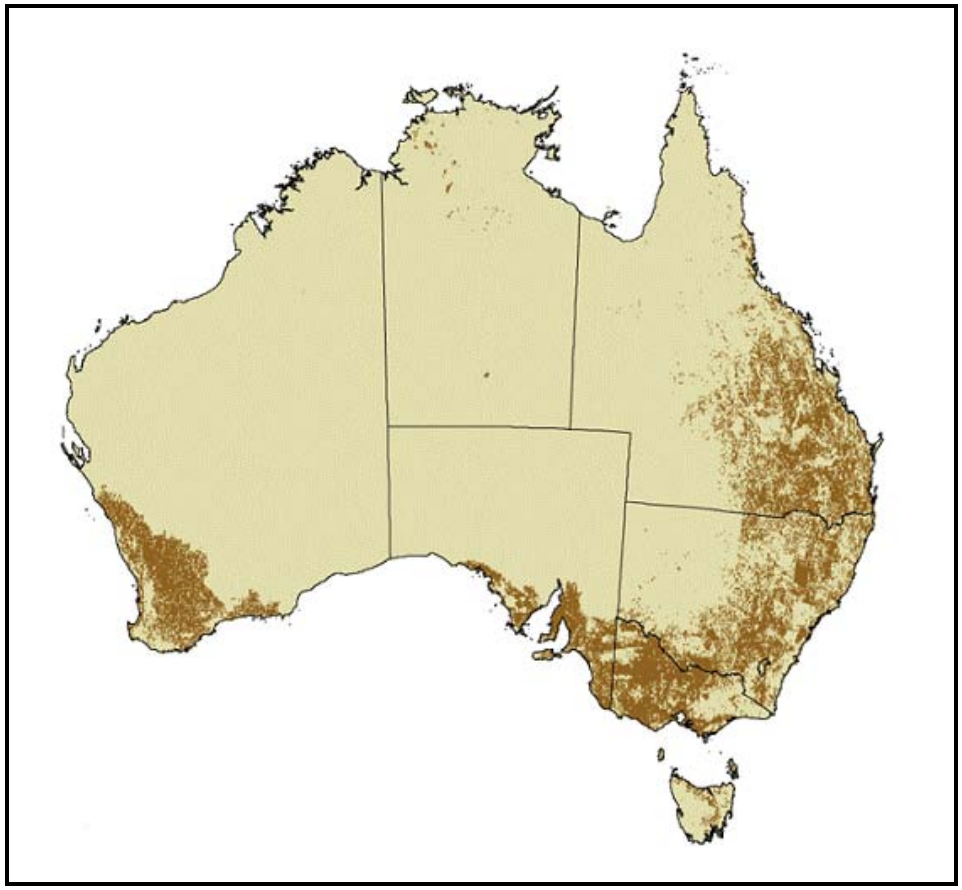

Figure 2.1 The extent of native vegetation clearance (in brown) in Australia as at 2001 (NLWRA 2001).

Salinisation

A vast amount of salt is present in the Australian landscape (Lambert and Turner 2000; Bennett and Virtue 2003). Most of the salt has been carried on the air from sea waves and deposited with precipitation, or originated from the weathering of mineral rocks liberated from old sea-bed material left when the land was covered by ocean (Stirzaker et al. 2002). This salt has progressively leached from the surface through the soil profile and accumulated in the regolith at soil depths of 30 m or more (McFarlane and George 1992).

Before its extensive clearance, the deep-rooted, woody perennial vegetation of southern Australia either evaporated or transpired the majority (up to 99%) of rainfall received (Nulsen et al. 1986; Taylor and Hoxley 2003) and only a small proportion was converted to surface runoff entering waterways (<0.5%) or recharged to groundwater (<0.5%) (Nulsen et al. 1986). As a result, ground water remained at depth, typically >30 m below the surface (Hatton et al. 2003; Taylor and Hoxley 2003).

With the removal of native vegetation a dramatically increased amount of water reaches the soil surface (Williamson 1998) and recharges to groundwater (Farrington et al. 1992) resulting in a rise in water tables to the soil surface. Along with the water, salt accumulated in the soil is mobilised and concentrated at the surface as the water evaporates. Alternatively, salinised groundwater discharges into water sources (Spies and Woodgate 2003). This hydrological imbalance is perpetuated by the presence of the shallow rooted winter active annual crops and pastures of traditional European farming practices as they intercept and transpire much less rainfall than the original perennial vegetation (Clarke et al. 2002).

Extent of Salinisation

Increasing salinisation of landscapes and waterways and its impacts are forecast to be particularly severe where vegetation clearing for agriculture has been the most extensive, as shown in Figure 2.2 (Hatton et al. 2003). At least 5% of all of Australia's agricultural land (2.5 million hectares) was estimated to be affected by dryland salinity in 2000 (COAG 2000) with the majority of that occurring over the extensively cleared southwest of Western Australia (ADSA 2001). Most of Australia's waterways are already subject to the impacts of salinisation (NLWRA 2001) and almost all of the rivers and wetlands in agricultural areas in Western Australia are in advanced states of salinisation (Schofield et al. 1989; Hatton and Ruprecht 2001). The area of land at risk of salinisation is expected to rise to 17 million hectares by 2050 at the current rate of increase (NLWRA 2001).

NOTE: This figure is included on page 8 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.2 Areas of land forecast to be of high hazard or risk of dryland salinity (in green) in 2050 (NLWRA 2001).

Impacts of Salinisation

The impacts of salinising land and waterways are dramatic, complex, and widespread. Increasing levels of salinisation are expected to be responsible for significant further direct declines in both compositional biodiversity and functional biodiversity (Keighery 2000; Hatton et al. 2003). Rural communities will be further affected by the degradation of infrastructure, reduced farm production profits and value, the retirement of land from agriculture, increased health problems, the exacerbation of pre-existing social problems, community division and declining populations, businesses and services (Pannel et al. 2001). These impacts on the environment, agricultural productivity and society will also involve substantial domino effects that are difficult to predict or quantify. Due to the complexity of the present and future impacts of dryland salinity, attempts to economically quantify the impact vary greatly (Short and McConnell 2000).

Land Use Change

Addressing Salinity

Several management options that attempt to address the increasing salinisation of Australia's agricultural landscapes have been proposed. These include engineering solutions that involve pumping and drainage to manage saline groundwater, and

interceptor banks for the diversion of saline local discharge (Beresford et al. 2001). However, engineering solutions require significant investment in construction and maintenance and offer only localised solutions simply diverting saline effluent to alternative areas and possibly concentrating pesticides, fertilisers, heavy metal and nutrients in the process (Ghassemi et al. 1995). Accepting salinity and utilising saline land for the economic production of algae, fish products or livestock feed for animal maintenance, and the extraction of minerals from saline land are options that have also been suggested (Pannel et al. 2001).

Obviously, none of these options will address the ongoing environmental impacts of increasing salinisation (Beresford et al. 2001; Pannel et al. 2001). More importantly, these management options are unsustainable, as they make no attempt to remediate salinity or even stem its increase by addressing the root cause - the hydrological imbalance brought about by extensive removal of vegetation in agricultural industry. The only long term answer that addresses this problem is the reintroduction of perennial plants on a large scale throughout Australia's agricultural landscapes (Clarke et al. 2002).

Agroforestry

It has been suggested that in order to restore essential functional ecosystem processes and attempt to address the hydrological imbalance and resulting salinisation, a 'revolution in land use' is required in the Australian agricultural landscape (Williams and Saunders 2003). This is essentially recognition of the need to modify traditional European agricultural practices to incorporate the large-scale use of perennial farming systems (Clarke et al. 2002).

If revegetation with perennials is to occur on a scale sufficiently large enough to address the salinity problem, new perennial farming systems that do not impact significantly on agricultural productivity must be developed (Pannel et al. 2001; Bartle et al. 2002). Primary consideration has therefore been given to systems that will be commercially competitive with the long-term average returns from traditional annual crops and pastures, and which can be easily incorporated into the traditional agricultural landscape (Pannel et al. 2001). It is this commercial component that separates new perennial

farming systems from traditional revegetation programs aimed at restoring biodiversity. Hence, the salinity problem is now largely seen as 'a need to modify the suite of commercial crop plants within agriculture, not only as an opportunity for public investment to restore modest areas of bush on farms' (Olsen et al. 2004).

The most favoured approach for perennial farming that will meet the requirements of scale and profitability involves the further development of perennial crop species and their large-scale use in farm forestry and agroforestry. '*Farm Forestry*' or the management of trees for a commercial purpose, is a subset of '*agroforestry*', which is generally thought to encompass a broader integration of perennial shrub and tree crops into agriculture to produce forest products and to benefit farm productivity and sustainability (Black et al. 2000).

Perennial Crops

Woody perennial species have a number of advantages over traditional European annual or pasture species. Large woody perennials have a greater capacity to restore the hydrological imbalance and halt increasing salinisation as they can intercept more rainfall, have more extensive root systems that can extract water from deeper and larger soil volumes, and make use of out of season (summer) rainfall (Farrington et al. 1992). They provide greater erosion control by contributing to deeper and more stable surface soil, do not need to be replanted, are more resistant to seasonal climate variation, can help diversify farm incomes and, through the opportunity for multiple harvests, provide more resilience in farm income, and have greater amenity value (Bartle 1991; Lefroy and Stirzaker 1999; Pannel et al. 2001). Revegetation using woody perennial plants will also provide a range of other ecosystem and off-farm benefits other than recharge control such as carbon sequestration and better protection for water catchments.

There are currently few economically attractive woody perennial options for the lower rainfall areas of southern Australia (250 – 650 mm annual rainfall) (Bathgate and Pannel 2002). Large-scale agroforestry in these low rainfall areas requires the development of new woody perennial crop species that produce a mix of saleable low value, bulk biomass products and that have markets with the ability to absorb large production volumes (Bartle 2001; Bartle et al. 2002). It is envisaged that woody perennial crops produce products such as small dimension timber and composite wood

products, residue and biochemical products such as essential oils and tannins, charcoal, activated carbon, livestock feed and bulk biomass for energy production (Bartle 2001; Bartle et al. 2002; Bartle and Shea 2003; Maslin and McDonald 2004; Olsen et al. 2004).

Native Species

The evaluation of new perennial crop species has focussed on natives as an added tactic for the protection of Australian biodiversity (Olsen et al. 2004). Native species are less likely to become invasive in comparison to exotics and are generally thought to have little potential for invasiveness when planted within their natural range. Unlike exotics, the full complement of genetic resource is available for exploitation and they have the added bonuses of being adapted to a range of sites in the harsh conditions and nutrient deficient soils of southern Australia, being easily established, and showing rapid growth and tolerance of low input management regimes (Bartle 2001; Olsen et al. 2004). Native species may also be better suited to maintaining ecosystem processes and supporting species diversity, for example by providing more favourable habitat for native fauna than exotic woody crops (Olsen et al. 2004).

Species Evaluation

Projects including the W.A. Search Project, Acacia Search and Flora Search have aimed to identify priority species for further development as agroforestry crops in the low rainfall areas of southern Australia (Bennell et al. 2004; Maslin and McDonald 2004; Olsen et al. 2004). A number of native species that show promise for further development have been identified including species of Eucalypts, Melaleucas, Acacias, Casuarinas and Grevilleas. In these studies, *Acacia saligna* has been identified as the priority species warranting further development.

Acacia saligna

Species Description

Acacia saligna (Labill.) H.L.Wendl. is also known as Koojong or the Golden Wreath Wattle. It is a leguminous species belonging to the former subgenus *Phyllodineae*. Botanical descriptions are given in Maslin (1974), Kodela (2002) and Doran and Turnbull (1997) among others. The most recent species description is given by Maslin (Maslin 2001) and follows: *Acacia saligna* (Labill.) H. Wndl., *Comm. Acac. Aphyll*. 4, 26 (1820). Bushy shrub or tree, 2-6 m high. Bark grey. Branchlets often pendulous,

normally slightly flexuouse, often pruinose (especially when young), glabrous. Phyllodes, often pendulous, variable in shape and size, linear to lanceolate, straight to falcate, 7-25 cm long, (2-) 4-20 mm wide, often large towards base of plant, green to glaucous, glabrous, with prominent midrib, finely penninerved (absent on very narrow phyllodes); gland +/- disciform, 1-2 mm wide, 0-3 mm pulvinus, mostly 1-2 mm long, coarsely wrinkled. Inflorescences mostly 2-10 headed racemes, enclosed when young by inbricate bracts, with bract scars evident at anthesis; raceme axes mostly 3-30 mm long, glabrous; peduncles 5-15 mm long, glabrous; head globular, mostly 7-10 mm diam. at anthesis and 25-55 flowered, golden. Flowers 5-merous; sepals c. 4/5-united. Pods linear, flat, shallowly constricted between seeds, 8-12 cm long, 4-6 mm wide, thinly coriaceous, glabrous. Seed longitudinal, oblong to slightly elliptic, 5-6 mm long, shiny, dark brown to black; aril clavate.

Occurrence

Acacia saligna is native to the south west of Western Australia where it is common with a widespread but discontinuous range. The species distribution, including both natural and planted populations, is shown in Figure 2.3. Over its natural range *A. saligna* experiences a Mediterranean climate of annual rainfall of between 300 and 1200 mm, falling mainly in the winter months. The species grows in a variety of habitats and occurs in association with a variety of plant communities typically in more open parts of dry sclerophyll forest, temperate woodlands, semi-arid woodlands and mallee (Fox 1995; Doran and Turnbull 1997). It shows a preference of deep sandy soils although will grow on a variety of soils. In many areas it favours creeks, rivers and their adjoining lands (Fox 1995).

Acacia saligna has been extensively planted in southwest Western Australia and can now be found outside of its presumed natural range (Fox 1995). The species has also been widely cultivated throughout southern and eastern Australia and is naturalised in South Australia, Queensland, New South Wales, Victoria and Tasmania (Muyt 2001). The species naturalised distribution in the eastern states is shown in Figure 2.4. *Acacia saligna* has also been planted extensively in semi-arid areas of Africa and the Middle East, including Ethiopia, Namibia, Tanzania, Kenya, South Africa, Tunisia, Morocco, Algeria, Egypt, Libya, Israel, Iran, Iraq, Jordan and Syria (NAS 1980; Crompton 1992; Doran and Turnbull 1997). The species has been introduced to the United States of

America and South America, for example to Uruguay, Mexico and Chile, and to European countries of France, Greece, Spain, Portugal, Turkey, Corsica and Cyprus (Midgley and Turnbull 2003; Maslin and McDonald 2004). It can also be found in India and New Zealand (Chakrabarty and Gangopadhyay 1996).

NOTE: This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.3 The distribution of *Acacia saligna* in Western Australia. Image used with the permission of the W.A. Herbarium, Department of Environment and Conservation.

Utilisation

Acacia saligna has a wide range of uses both in Australia and overseas. The species has been used as a source of yellow dye, as an ornamental species, for shade, fodder and firewood, for erosion control, sand dune stabilisation, reduction of nutrient runoff, mine site rehabilitation, and as a windbreak and visual screen (Crompton 1992; Fox 1995;

Maslin 2001; Midgley and Turnbull 2003; Maslin and McDonald 2004; Maslin et al. 2006). It has been extensively planted across southern Australia for mine site rehabilitation and revegetation purposes as well as for amenities plantings and is listed as a plant of economic importance to Australia by Shepard et al. (2001).

NOTE: This figure is included on page 14 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.4 The naturalised distribution of *Acacia saligna* in Australia. Image used with the permission of Australia's Virtual Herbarium.

With a short life span of up to 20 years, *A. saligna* shows promise as a short rotation phase or coppice crop in the low rainfall agricultural areas of southern Australia (Bartle et al. 2002). Being a pioneer species, the large, hard-coated seed germinates readily and is amenable to direct seeding. Trees grow rapidly with good survival and can improve the soil through nitrogen fixation (Scheltema 1992). The trees are tolerant to drought, alkalinity, salt and light frost (Simmons 1987). In Mediterranean countries the species is harvested for fuel wood on a coppice rotation system of 5-10 years (NAS, 1980) and a similar cropping system has been recommended for the species in southern Australia (Michaelides 1979). Some subspecies show coppicing ability and it is also possible that shallow ploughing to induce root suckering could rejuvenate crops (Michaelides 1979).

Intersubspecific Variation and Distribution

Acacia saligna is highly variable in morphological, ecological and biological attributes over its natural distribution. Presently it is considered a species complex that is inadequately described taxonomically. Recent studies have the species complex accommodated within four morphological subspecies illustrated in Figure 2.5, the latest descriptions of which are provided by Masin and McDonald et al. (2006) and McDonald and Maslin (in prep) and will be summarised here. Records of the distribution of the four subspecies in Western Australia are illustrated in Figure 2.6, although this figure includes reports of both natural and planted populations.

Subspecies *lindleyi* (previously informally referred to as "Typical" variant) has the most widespread distribution of the subspecies, occurring across the inland wheatbelt areas and on southern sand dunes. Trees are commonly 2-5 m tall with straight stems, smooth bark, and green phyllodes. Suckering propensity is low. Trees are often spindly with little biomass and terminal flowering limited to the end of branches. Subspecies *lindleyi* usually has slightly shiny to shiny adult phyllodes (particularly new shoots), that lack a notably attenuated base; a conical inflorescence bud, 0.4-0.7 cm long; often 5-headed racemes, up to 4 cm long; and light golden flower heads, that are 1.2-1.3 cm in diameter when fresh.

The *saligna* subspecies (previously informally referred to as "Cyanophylla" variant) is geographically restricted largely to the Swan Coastal Plain around Perth although populations have also been located on the southern coast near Albany and Esperance. It grows to 3-10 m tall, has a robust main stem, smooth, reddish bark, and relatively narrow phyllodes. Trees are dense and bushy with good biomass production, coppice well and sucker less profusely. Subspecies *saligna* has dull adult phyllodes with a notably attenuated base; an obtuse inflorescence bud, 0.2-0.3 cm long; often 7 to 8-headed racemes, up to 6 cm long; and yellow to light golden flower heads, that are 1.7-1.8 cm in diameter when fresh. This variant has been commonly used as an ornamental and in revegetation programs and is likely to be the variant most commonly found in the eastern states.

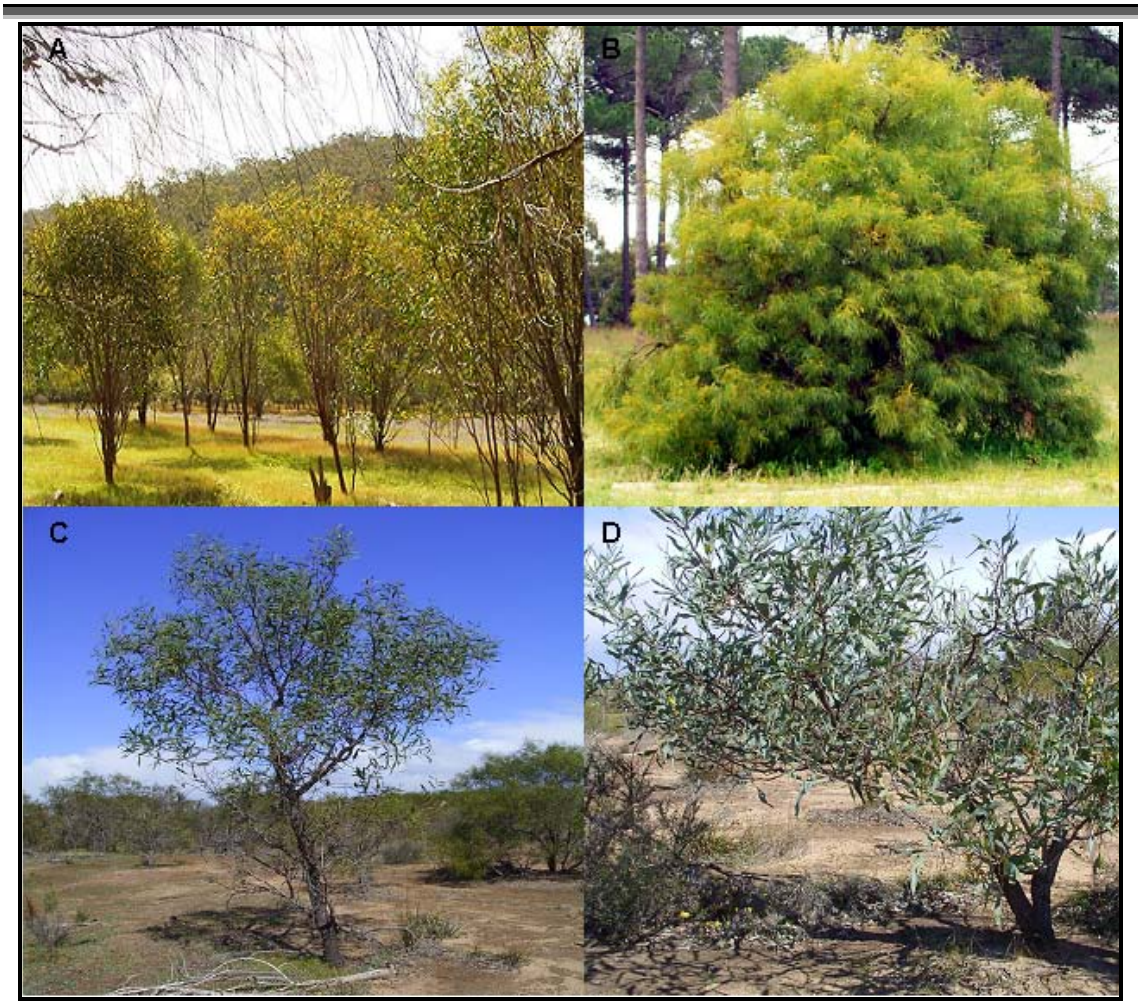

Figure 2.5 The four main subspecies of *Acacia saligna* (McDonald and Maslin, in prep; Photography MA Millar). Mature specimens of subspecies (A) *lindleyi* (B) *saligna* (C) *stolonifera* and (D) *pruinescens*.

The natural distribution of subspecies *stolonifera* (previously informally referred to as "Forest" variant) is from the Swan Coastal Plain south to Walpole and east toward the range of subspecies *lindleyi*. Its distribution has considerable overlap with that of subspecies *pruinescens*. It grows 3-10 m tall, usually with a single stem, which may be crooked and has friable bark. Trees sucker strongly. Subspecies *stolonifera* has dull adult phyllodes which lack a notably attenuated base; a conical inflorescence bud, 0.4-0.7 cm long; commonly 5-headed racemes, up to 2.5 cm long; and pale yellow flower heads, that are 1.7-1.8 cm in diameter when fresh.

Subspecies *pruinescens* (previously informally referred to as "Tweed River" variant) occurs from Williams and Collie south to Bridgetown-Kojonup-Manjimup although populations have also been reported to the east and north east of Esperance. Trees grow

3-10 m tall with typically large, crooked, white pruinose stems, friable bark and glaucous leaves. This variant also suckers strongly. Subspecies *pruinescens* has bluish, dull, lightly pruinose adult phyllodes (adolescent foliage is densely pruinose and up to 10 cm wide); a conical inflorescence bud, 0.4-0.7 cm long; usually 7 to 8-headed racemes, up to 5.8 cm long; and yellow to light golden flower heads, that are 1.4 cm in diameter when fresh.

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.6 The Western Australian distribution of four proposed subspecies of *Acacia saligna* (McDonald and Maslin, in prep). Image used with the permission of the W.A. Herbarium, Department of Environment and Conservation.

A fifth subspecies, subspecies *osullivaniana*, of restricted range north of Perth, has also recently been described by McDonald and Maslin (in prep). Its relationship to other taxonomic entities is unknown. It is a sparsely-foliaged, short-lived shrub,

distinguished by its dull, spreading, linear adult phyllodes (0.2-0.4 cm wide); inflorescence bracts that shed early (not forming a resting inflorescence bud phase); 7-headed racemes, up to 5 cm long; and yellow to light golden flower heads, that are 1.2-1.4 cm in diameter when fresh.

The main morphological characteristics that form the basis of subspecies identification are differences in the shape of the floral bracts, the size and colour of inflorescences and the number of flowers in the inflorescence (McDonald and Maslin in prep) and work is currently being undertaken to discriminate the subspecies by coding their differences into an electronic key and to formally rank and name the taxa (Maslin et al. 2006).

Species Development for Sustainable Agroforestry

The Department of Environment and Conservation (formerly the Department of Conservation and Land Management) began serious investigation of the commercial potential of Koojong in the late 1990s. Options for potential bulk commodity, low value biomass products for *A. saligna* have been assessed and are likely to include panel boards and other wood products, bioenergy, fodder and biomaterials and chemicals. Further development of harvesting techniques and equipment, transport systems, markets, and integrated processing plants is required to make these options viable (Olsen et al. 2004).

A number of mixed species field trials were established by the Department of Environment and Conservation between 2000 and 2004, which included several provenances of *A. saligna*. In addition, a number of spacing trials using *A. saligna* were established in 2003 with the objective of measuring biomass production and water use (Huxtable et al. 2006). Based on the initial results of these trials, a domestication program commenced in 2004 when extensive germplasm collections were made and provenance trials consisting of seedlings representative of 20 provenance collections were planted at two sites in W.A. in 2006. The domestication program will involve intensive field performance testing, ongoing progeny testing, and selective breeding to improve traits such as height, stem diameter, survival, form, biomass production and feedstock and forage characteristics (Huxtable et al. 2006).

Knowledge Gaps

Despite its long and varied history of use, there are a number of knowledge gaps and issues associated with the development of the *A. saligna* species complex as an agroforestry crop for the southern agricultural regions of Australia. Information is required in a number of key areas to aid both the ongoing domestication and breeding program and for the conservation of genetic diversity in the species complex across its natural range. A number of these knowledge gaps are addressed in the work of this thesis and relate to:

- the reproductive system
- patterns of gene flow
- structuring of genetic diversity
- subspecific population identification.

Reproductive system

The reproductive system encompasses aspects of a species floral biology, floral phenology, mating system and patterns of pollen dispersal. An efficient breeding and domestication program for *A. saligna* will require detailed knowledge of these aspects of the reproductive system. Knowledge of floral biology and the relative floral phenologies of subspecies of *A. saligna* will be required for successful controlled crossing. Aspects of floral biology including the small hermaphrodite flowers grouped closely together in spherical inflorescences, stigmas surrounded by a large number of pollen bearing anthers, and little time between stigma reception and anther dehiscence (George 2005) make emasculation and controlled pollination difficult in *A. saligna*. In addition, pollen has been shown to lose viability after collection and storage (George 2005).

Knowledge of relative floral phenologies, the mating system and patterns of pollen dispersal can be used in the design and management of populations planted for seed production such as seed orchards (El-Kassaby 2000). Seed orchards are established to produce commercial quantities of easily harvestable seed from selected superior trees or trees improved by breeding. Most tree seed orchards are open pollinated and established by setting out clones or seedling progeny of trees selected for desired

characteristics (Kang et al. 2001). However, in the early stages of the *A. saligna* domestication program stands originally planted as provenance trials would be suitable for conversion to seed orchards by selective thinning and leaving the best trees for seed production.

Seed crops harvested from seed orchards must be of high genetic value; that is, they should capture a large amount of the genetic diversity present in genotypes within the orchard (Kang et al. 2001). This requirement is met by ensuring that maternal and paternal reproductive contributions of all trees are equal, high levels of outcrossing are achieved and patterns of pollen dispersal and mating are random among the genotypes within the orchard (Friedman and Adams 1985; Adams et al. 1997). In seed orchards comprised of more than one subspecies, such as those developed from provenance trials, these aims will be achieved when inter-subspecific crossing or hybridisation within the orchard is maximised.

Patterns of inter-subspecific mating in open pollinated seed orchards of *A. saligna* will be dependent on the levels of interfertility and overlap in floral phenologies of the subspecies. The subspecies of *A. saligna* must be suitably interfertile or cross compatible for inter-subspecific hybridisation to occur. Although it has not been investigated in detail, there is genetic evidence of hybridisation between the subspecies (George et al. 2006). Differences are apparent in the peak flowering times of the subspecies although there is overlap. The species flowers mostly from late July to October. Over their natural distributions, subspecies *lindleyi* and subspecies *stolonifera* show peak flowering in mid October, subspecies *saligna* has the earliest and most prolonged flowering period lasting from August to September, while subspecies *pruinescens* flowers in late September (Maslin et al. 2006). The overlap in floral phenologies suggests that pollen transfer will be possible between the subspecific entities and that inter-subspecific crossing may occur in seed orchards comprised of more than one subspecies.

Investigations of the mating system for a number of *Acacia* species, using isozymes, indicate mixed mating systems with variable levels of outcrossing: mean multilocus outcrossing rates ranging from approximately 60% to over 90% (Moran et al. 1989b; Muona et al. 1991; Mandal and Ennos 1995; Casiva et al. 2004). A recent isozyme

study of three natural populations and one cultivated mixed provenance population of *A. saligna* indicated a mixed mating system with preferential outcrossing, with a mean multilocus outcrossing rate of 0.87 and a range of 0.80 to 0.99 (George 2005). The estimated mean multilocus outcrossing rate for the planted population was 0.92. These results indicate that it should be possible to manage seed orchards comprised of multiple subspecies of *A. saligna* to ensure high levels of outcrossing.

Reduced outcrossing rates or increased levels of inbreeding in seed production stands of *A. saligna* will result in decreased genetic diversity in subsequent seed crops (Friedman and Adams 1985; Wheeler and Jech 1992). Increased inbreeding may also result in reduced levels of seed production and reduced vigour in progeny via inbreeding depression, a reduction in fitness due to increased homozygosity (Edmands 2007). Inbreeding depression is evident in *Acacia* where selfed progeny show lowered viability and reduced vigour (Kenrick and Knox 1989a; Butcher et al. 1999; Harwood et al. 2004) and high levels of inbreeding have been proposed as the cause of the poor performance of a seed production stand of *A. mangium* (Butcher et al. 1999). Outcrossing rates, levels of inbreeding and the impacts of inbreeding depression are largely a result of pre- and post-zygotic self-incompatibility mechanisms which have been reported at varying degrees in most *Acacia* species studied (Kenrick 2003).

Levels of outcrossing, and the extent to which mating approaches random within a seed orchard, are also determined by patterns of pollen dispersal. Localised patterns of pollen dispersal and the degree of heterogeneity in pollen clouds experienced by maternal trees, or the extent of panmictic mating, have not been investigated in *A. saligna* although such information would be of value in the design and management of seed orchards.

Patterns of pollen-mediated gene flow

Aspects of the reproductive system and patterns of pollen dispersal also affect the likelihood of pollen-mediated gene flow into seed production stands. Pollen-mediated gene flow into seed orchards from genetically divergent populations such as remnant natural populations will act to dilute the superior genotypes in subsequent seed crops (Wheeler and Jech 1992; Adams et al. 1997). Seed orchards must therefore be managed

to minimise genetic contamination from pollen from inferior trees located outside the stand.

Pollen-mediated gene flow from stands of *A. saligna* planted for agroforestry purposes, be they seed production stands or commercial crops, may also present a threat to remnant natural populations. Genetic contamination via pollen-mediated gene flow may occur in remnant populations of *A. saligna* when large stands of genetically divergent material from domesticated or non-local populations are planted for agroforestry within their natural range. This may result in negative consequences for the maintenance of genetic diversity and the long-term persistence of natural populations (Rhymer and Simberloff 1996).

Long term pollen-mediated gene flow is usually considered beneficial to plant conservation being an important factor in natural population and ecosystem processes (Ellstrand and Ellam 1993). Gene flow shapes gene pools and the population genetic structure of species. It acts as a force to maintain genetic continuity between populations, counteracting the effects of mutation, drift and selection (Ellstrand et al. 1999a) and preventing the loss of genetic diversity through inbreeding. Gene flow may become detrimental however, when it occurs between genetically divergent populations (Ellstrand et al. 1999a). Pollen-mediated gene flow between genetically divergent populations may result in the production of hybrid progeny and the term hybridisation describes interbreeding among genetically divergent populations, regardless of their taxonomic status (Rhymer and Simberloff 1996). Both the likelihood of gene flow between agroforestry plantings and natural populations, and the potential impacts of hybridisation in natural populations of *A. saligna* need to be assessed.

Likelihood of genetic contamination

The likelihood that genetic contamination will occur between genetically divergent populations of *A. saligna* is difficult to predict but is directly related to patterns of pollen dispersal. In tree species, patterns and distances of pollen dispersal are affected by the type of pollen dispersal and behaviour of the pollination vector, however dispersal is highly idiosyncratic and can vary greatly in different environments at different times and even for different individuals (Slatkin 1987).

Early studies of pollen-mediated gene flow were conducted largely for wind pollinated species and nearly always describe a leptokurtic distribution of pollen from its source, with the majority of pollen travelling a short distance and a thin tail of long distance distribution (Levin and Kerster 1974; Ellstrand 1992). However, the extent of the tail of the dispersal curve is important and recent studies have shown long distance pollen dispersal is often underestimated and extensive in woody species with bird, mammal and insect pollination vectors (Hamrick and Murawski 1990; Stacy et al. 1996; Konuma et al. 2000; Dick 2001; Dick et al. 2003; Latouche-Halle et al. 2004). Acacia species are generally pollinated by a range of vectors including bees, other insects and birds (Sedgley et al. 1992) and a study of possible pollinators in *A. saligna* recorded many insect species from the Hemiptera, Coleoptera and Diptera orders and the European honeybee *Apis mellifera* visiting flowers (George 2005). These bees and small generalist insect pollinators are capable of effecting long distance pollen-mediated gene flow (Levin and Kerster 1969; White et al. 2002).

Recent studies have also shown that population fragmentation leads to increased gene flow particularly for scattered trees and populations (Nason and Hamrick 1997; Dick 2001; White et al. 2002; Bacles et al. 2005). This is a factor in the Australian landscape and recent analysis of gene flow in fragmented populations of *Eucalyptus wandoo* in the W. A. wheat belt revealed a fat-tailed pollen dispersal curve with substantial levels of long distance pollen dispersal, with 50% of the pollen load of some trees coming from greater than 1 km away (Byrne et al. 2007). The combination of a highly outcrossed mating system, entomophilous pollinators and highly fragmented populations indicates that similar high levels of long distance pollen dispersal may be expected in *A. saligna*.

Impacts of pollen dispersal

The consequences of pollen-mediated gene flow among genetically divergent populations and the production of hybrid progeny have not been investigated for *A. saligna* but hybridisation typically results in one of two outcomes for hybrid progeny: heterosis or outbreeding depression (Ellstrand and Ellam 1993).

Heterosis, or hybrid vigour, is said to occur when hybrid fitness is enhanced relative to genotypes in the parental populations and is attributed to overdominance or the masking of recessive deleterious alleles (Lynch 1991). The increased vigour of an initial hybrid

generation may result in the establishment of large numbers of hybrid progeny in remnant populations of *A. saligna*. If hybrids maintain increased fitness relative to parental genotypes in the remnant population through successive generations, then pure progeny of the remnant population may be at risk from direct competition. If hybrid fitness is displayed in increased gamete production and fertility, then the parental population may be at risk from hybrid 'swamping' or introgression (Huxel 1999). Genetic swamping by large non-native plantings (Ellstrand and Ellam 1993) has been reported as threatening several forest tree species or provenances including poplar (Smith and Sytsma 1990; Cagelli and Lefevre 1995), fir (Mejnartowicz 1996), mahogany (Rieseberg and Gerber 1995) and sycamore (Rhymer and Simberloff 1996).

Introgression is the transfer of genes from one population to another by the repeated backcrossing of hybrids to one or both of the parental populations (Rhymer and Simberloff 1996). Introgression may result in a small component of the donor genome, typically that of the larger population, being transferred to the smaller population, or may extend to virtually complete introgressive displacement of the genome in the smaller population (Huxel 1999). Continued hybridisation with a large stand planted for agroforestry purposes may eventually result in the total genetic assimilation of a small remnant population via introgression. In the absence of selection against hybrids, such genetic assimilation or displacement results in the rapid extinction of small remnant populations (Rhymer and Simberloff 1996). Hybridisation is not necessarily accompanied by introgression, as may be the case when hybrids display outbreeding depression or when all hybrids produced are sterile.

The other possible outcome for hybrid progeny is outbreeding depression. This occurs via unfavourable genomic interactions and is observed as a reduction in hybrid fitness relative to the genotypes in the parental population (Huxel 1999). Outbreeding depression may occur in hybrid progeny in different ways depending on the nature of the genetic divergence between the parental populations (Templeton 1986; Edmands 2007). If fitness in a remnant population has occurred through selection for locally adapted genotypes, then hybridisation will result in the dilution of these genotypes, as hybrids show heterozygosity and underdominance at loci formerly fixed for adapted alleles (Templeton 1986). If fitness in the remnant population has occurred via the development of co-adapted gene complexes (distinct combinations of epistatically

interacting loci), then these complexes may be exposed to disruption though recombination in hybrid progeny (Edmands 2007). Evidence of outbreeding depression is often not seen until the F2 generation where it is described as advanced generation breakdown (Templeton 1986; Fenster and Dudash 1994; Hufford and Mazer 2003).

The increasingly fragmented agricultural landscapes of southern Western Australia are typically composed of small remnant populations, where gene flow is especially important in shaping genetic structure. Large-scale changes in gene flow are likely to have negative consequences for the long-term persistence of small native populations including a loss of genetic integrity and reduced adaptive potential (Huxel 1999; Rhymer and Simberloff 1996), especially as remnants may already be suffering from the effects of small population processes, such as inbreeding depression, that produce a significant decrease in reproductive performance (Ellstrand and Ellam 1993). Regardless of their relative fitness, the production of hybrid progeny will result in further reduction to reproductive output in terms of pure progeny in small remnant populations of *A. saligna*. Model systems have shown that the level of successful reproduction in a remnant population may be rapidly lowered to a point that leads to displacement even when hybrids are sterile (Kondrashov 1992; Huxel 1999; Wolf et al. 2001).

The impacts of hybridisation, as a result of pollen-mediated gene flow in *A. saligna*, are difficult to predict, but will also depend on the scale and type of genetic divergence between the domesticated or non-local and natural populations. The greater the level of genetic divergence between two populations the greater the effects of outbreeding depression are likely to be (Edmands 2007). The *A. saligna* domestication and breeding program should have captured a broad base of genetic diversity initially. In addition, the species has undergone no selective breeding for domestication to date, so breeding itself will have contributed little toward genetic divergence of 'domesticated' and natural populations. However, the *A. saligna* breeding program aims to select for traits such as survival, growth form and biomass production. Being a species complex, these characteristics may be more predominant in a single subspecies and the breeding program may focus on those subspecies displaying important agronomic traits. At this stage, subspecies *saligna* and subspecies *lindleyi* generally display the best traits for an agroforestry crop, however this will become apparent only when trees are grown under

the conditions, and in the areas intended, for agroforestry. Genetic diversity within the breeding germplasm will also become reduced with repeated selection for superior genotypes and domesticated populations utilised for agroforestry may then diverge significantly from that of natural populations.

Structuring of genetic diversity

Knowledge of the genetic entities and the levels of genetic diversity between subspecific entities in *A. saligna* will not only provide information on the likely impacts of hybridisation via pollen-mediated gene flow within the species complex but is also a basic requirement for effective domestication and breeding. Genetic studies have been undertaken on a number of *Acacia* species domesticated throughout the world for wood, seed, fodder, tannin and cut flower production. Moderate to high levels of geographically structured population differentiation have been identified in the tropical species *Acacia mangium* (Moran et al. 1989a; Butcher et al. 1998), *A. auriculiformis* (Wickneswari and Norwati 1993), *A. tumida* (McDonald et al. 2003) and *A. aulacocarpa* (McGranahan et al. 1997). Genetic structuring into geographic regions was also identified in the temperate species *A. melanoxylon* (Playford et al. 1993), but not in temperate *A. mearnsii* (Searle et al. 2000).

In species complexes, where genetic structuring has been detected, taxonomic revision has led to the description of previously unidentified taxa. For example, five taxa were identified in the native Queensland species complex *Acacia aulacocarpa* (McDonald and Maslin 2000). Combined genetic and taxonomic approaches have also been undertaken to resolve the ambiguities in a number of species complexes identified for development for agroforestry in Western Australia (Byrne 1999; Byrne and Broadhurst 2003; Broadhurst et al. 2004). In the *Melaleuca uncinata* species complex, which is commercially harvested for broombush fencing, eleven species have been identified across the natural range in the south west of Western Australia (Craven et al. 2004). Distinct taxonomic entities have also been identified by investigations of the genetic relationships among recognised variants in *Acacia microbotrya* and *A. acuminata*, both species of interest for development for agroforestry (Byrne 2002; Byrne and Broadhurst 2003).

The *Acacia saligna* species complex shows a considerable level of genetic structuring although the genetic relationships between entities have not yet been fully resolved and further investigation is required. A molecular genetic study using Restriction Fragment Length Polymorphisms (RFLP) conducted by George et al. (2006) analysed genetic diversity for a number of natural populations across the species range. The authors found high population differentiation ($\theta = 0.349$) distributed evenly across populations and high genetic diversity for the species overall ($H_T = 0.417$ overall). Phylogenetic analysis indicated three genetically distinct groups separated by a high genetic distance (mean genetic distance between groups = 0.322). Two of the genetic groups corresponded to subspecies *lindley*i and subspecies *stolonifera* while the third group encompassed both subspecies *salign*a and subspecies p*ruinescens*, which were not genetically differentiated.

The RFLP study indicates strong inter-subspecific structuring but the failure to resolve the genetic relationship between subspecies *saligna* and subspecies *pruinescens* is inconsistent with the current taxonomic revision of the species. The results are also surprising as subspecies *saligna* and subspecies *pruinescens* are the most morphologically distinct and geographically separated of the subspecies (Maslin and McDonald 2004). Further work is required to fully elucidate the taxonomic entities and their genetic relationships in the *A. saligna* species complex.

Subspecific population identification

Resolution of the taxonomic entities within the *A. saligna* species complex will assist in delineating the natural distribution of each subspecies and in the identification of populations at the sub-specific level in the field. Because of the great morphological variation exhibited in *A. saligna* the accurate identification of populations in the field has often been a difficult task (Byrne and Broadhurst 2003). Population identification is hampered by morphological variation due to the biological age of the trees, which exhibit variable juvenile growth, and by changes in phyllode colour displayed over the seasons, and may be further exacerbated by phenotypic variation influenced by the specific growing environment. The main morphological characteristics that form the basis of subspecies identification are subtle differences in the shape of the floral bracts, the size and colour of inflorescences and the number of flowers in the inflorescence (McDonald and Maslin in prep). These characteristics are only observable at certain

developmental stages and hence at certain times of the year. In addition, the species has been planted extensively across its natural range, which makes the demarcation of the natural range of each subspecies and the delineation of natural and introduced populations difficult.

Accurate identification of populations of *A. saligna* at the subspecific level will be a first step for domestication and breeding programs, for the development of risk assessment strategies, and for research into gene flow, hybridisation and invasiveness. Accurate identification of populations enables better delineation of the natural geographic boundaries of subspecific taxa (Byrne and Broadhurst 2002). Knowledge of the geographic distributions of subspecific taxa can be used to ensure that germplasm collections, which will form the basis of the domestication and breeding program, are efficient and comprehensive in terms of capturing a large amount of genetic diversity (Brown and Brubaker 2000). Knowledge of subspecies distributions also allows for the development of appropriate *in situ* conservation and risk management strategies that may be required with large-scale use of the species for agroforestry. Accurate population identification is essential for studies of gene flow, hybridisation and introgression that may occur in areas where one or more subspecies are sympatric or where populations are introduced for agroforestry into the range of genetically divergent natural populations. Populations in areas outside the natural range, which are often invasive, are also typically of unknown origin. The identification of naturalised and invasive populations at the subspecific level will assist in determining whether a specific subspecies is more invasive than others, and in limiting the further introduction and spread of *A. saligna* as a weed in areas outside its natural range.

Despite extensive cryptic morphological variation, high levels of genetic differentiation among the different subspecies of *A. saligna* (George et al. 2006) indicate that a DNA based diagnostic tool allowing for the accurate characterisation of individuals or populations of *A. saligna* at the subspecific level could be developed.

Methodology for Genetic Analysis

One approach that could be used to further assess the mating system, patterns of inter- and intra-subspecific pollen mediated gene flow, the structuring of genetic diversity and in the identification of populations at the subspecific level for the *A. saligna* species

complex involves using highly polymorphic molecular markers. Isozymes or enzyme polymorphisms, and RFLPs which are based on DNA polymorphisms, have commonly been used for assessing mating systems, genetic diversity and population differentiation (Parker et al. 1998): isozymes originally as they are inexpensive and rapid, and later RFLPs as they show greater polymorphism and are less likely than isozymes to be subject to selection.

However, there is now a range of DNA-based molecular markers in use that exhibit even greater levels of polymorphism than isozymes or RFLPs. These include microsatellite markers otherwise known as SSRs (simple sequence repeats) or VNTRs (variable number tandem repeats), RAPDs (randomly amplified polymorphic DNA), SNPs (single nuclear polymorphisms), AFLPs (amplified fragment length polymorphisms), sequenced loci and others (Zhang and Hewitt 2003). These techniques have the added advantages of requiring less DNA and being quicker to perform than RFLP techniques.

In recent years the molecular markers of choice for the investigation of mating systems, and inferring genetic structure have been microsatellite markers. These are short tandem repeats of 1 to 6 base pairs found in the nuclear genome of all prokaryotic and eukaryotic organisms analysed to date (Zane et al. 2002). Microsatellite markers are codominant, obey Mendelian inheritance and are generally considered to be selectively neutral, although the last assumption is being increasingly brought into question due to the conservation across large evolutionary distances found for some loci (Zhang and Hewitt 2003). Nevertheless, microsatellites show a high degree of length polymorphism, most likely created by slippage events during DNA replication (Schlötterer and Tautz 1992), which makes them powerful genetic markers. In comparisons of several eucalypt species, diversity in microsatellite loci has been found to be twice as variable as RFLP loci and on average four times more variable than isozyme loci (Byrne 2007). Because of the high level of polymorphism, combined with significant advances in statistical techniques, microsatellite markers are now also routinely used to investigate pollen-mediated gene flow, and in the identification of populations at the subspecific level (Nybom 2004).

Once multilocus genotypic data has been collected for a number of maternal trees and a subset of their progeny using molecular markers, measures of the mating system, including the outcrossing rate and level of correlated paternity can be retrospectively inferred (Ritland 2002). Similar multilocus genotypic data on parental and progeny generations can be used for 'paternity analysis' or 'parentage analysis'. When multilocus genotypic data for a number of maternal trees and a subset of their progeny is combined with the multilocus genotypic data of possible paternal trees, parentages can be reconstructed and possible paternal parents excluded based on genotypic incompatibilities (Chakraborty et al. 1988). Statistical methods that assign a most likely pollen parent, rather than exclude non-parents, have more recently been developed and are based on the calculation of likelihood scores that compare the likelihood of an individual being the parent of a specific progeny, with the likelihood of that potential paternal parent and specific progeny being unrelated (Meagher 1986; Devlin et al. 1988). Maximum likelihood assignment methods can also assign a most likely paternal parent when there are many potential paternal trees and genotypic data is available for only a subset (Meagher 1986) and the technique has been further refined to include the calculation of statistical confidences in paternal assignments (Devlin and Ellstrand 1990; Marshall et al. 1998; Nielsen et al. 2001; Gerber et al. 2003).

When all trees within a selected area, such as a planted stand or remnant population, are genotyped, paternity analysis may produce a number of progeny that cannot be assigned a paternal parent from inside that population. These progeny are then assumed to be the result of pollen-mediated gene flow from outside the population and thus provide an estimate of gene contamination into the population (Dow and Ashley 1998; Valbuena-Carabana et al. 2005). When allele frequencies differ greatly for trees inside and outside the study population, as is likely if populations are comprised of different subspecies of *A. saligna*, then paternity analysis will be more effective and undetected cryptic gene flow (a result of similar alleles for each population) from outside the study population will be minimised (Gerber et al. 2003). When information on the specific location of maternal and assigned most likely paternal trees is obtained and mapped, detailed patterns of pollen-mediated gene flow can be described, including the precise distance over which pollen has travelled to sire each specific progeny (Sato et al. 2006; Byrne et al. 2007; Jones et al. 2007).

Multilocus genotypic data obtained with microsatellite markers can also be used to infer population structure in *A. saligna* using clustering methods (Manel et al. 2005). Bayesian model-based statistical methods that do not rely on *a priori* information regarding discrete genetic entities have recently been developed (Pritchard et al. 2000) and when multilocus genotypic data is obtained for individuals from populations that cover the species entire range, these methods can be used to divide the individuals into clusters using genetic criterion that define them as a group (Manel et al. 2005). Individuals are assigned to a group or cluster based on the assumption of Hardy Weinberg and linkage equilibrium within each randomly mating cluster (Pritchard et al. 2000; Pearse and Crandall 2004). Using this technique, the number of clusters and therefore the number of genetically distinct entities within the *A. saligna* species complex can be revealed (Evanno et al. 2005). The populations assigned to each cluster can then be compared to the taxonomic entities described by morphological analysis.

A similar approach could be used to develop a diagnostic tool for the accurate identification of populations of *A. saligna* at the subspecific level. If cluster analysis based on the assignment of individuals in a reference data set is congruent with the described subspecies, multilocus genotypic data for individuals of unknown or cryptic taxonomic classification can be compared to those in the reference data set (Manel et al. 2005). When prior information on the number of clusters in the reference data set is provided, cryptic individuals can be assigned to one or more of the clusters or subspecies with a known level of confidence (Paetkau et al. 1995; Rannala and Mountain 1997; Manel et al. 2002; Manel et al. 2005). Such a technique, being based on PCR (polymerase chain reaction), would be a rapid and cost efficient approach to take for the identification of cryptic individuals or populations (Duminil et al. 2006).

Because PCR is used to amplify microsatellite markers they can be used with fast and cheap DNA extraction methods (Selkoe and Toonen 2006). Unfortunately, being often found in non coding regions where the nucleotide substitution rate is higher than in coding regions (Zane et al. 2002), microsatellite markers are often species specific and need to be isolated *de novo* from the species of interest (Glenn and Schable 2005). Because *Acacia* is a large genus consisting of over 1000 species, it is unlikely that microsatellite markers developed in other *Acacia* species will show suitable levels of cross amplification for use in *A. saligna*. There are two published reports of

microsatellite markers developed in Australian *Acacia* species: one for *Acacia mangium* (Butcher et al. 2000) and one for the *Acacia mangium* x *Acacia auriculiformis* hybrid (Ng et al. 2005). These markers, being publicly available, could initially be tested for cross amplification in *A. saligna*, however, microsatellite markers may have to be developed specifically for *A. saligna*.

Addressing the knowledge gaps in *A. saligna*: Specific Objectives

In this thesis I use microsatellite markers to investigate and address a number of knowledge gaps associated with the utilisation of *A. saligna* as an environmentally sustainable agroforestry cultivar. The specific objectives of this study are:

- To develop highly informative microsatellite markers for *A. saligna*.

- To investigate the mating system and pattern of pollen dispersal for a planted stand of *A. saligna* using multilocus microsatellite genotypes.

- To investigate the level of inter-subspecific gene flow via pollen immigration (genetic contamination) into a remnant population and into a planted stand of *A. saligna* and the distance over which it occurs using paternity analysis of multilocus microsatellite genotypes.

- To investigate genetic structuring present within *A. saligna* and assess the congruence with taxonomic reclassification of the species complex.

- To develop a rapid and accurate PCR based molecular diagnostic technique for accurate identification of individuals and populations of *A. saligna* at the subspecies level.

Chapter Three
High outcrossing and random pollen dispersal in a planted stand of *Acacia saligna* subsp. *saligna* revealed by paternity analysis using microsatellites.

Statement of Contribution

M. A. MILLAR

Conducted the site selection, experimental design, all fieldwork, all laboratory work, all data and results analysis, wrote the final paper and acted as corresponding author

Signed Date

M. BYRNE

Provided assistance with theoretical conceptualisation, experimental design and writing the final paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

I. NUBERG

Reviewed and provided comment on final drafts of the paper

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

M. SEDGLEY

Reviewed and provided comment on final drafts of the paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

Chapter Three

High outcrossing and random pollen dispersal in a planted stand of *Acacia saligna* subsp. *saligna* revealed by paternity analysis using microsatellites

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Key words: microsatellite, mating system, pollen dispersal, male mating success, paternity analysis, Acacia saligna

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Abstract

The mating system, patterns of pollen mediated gene flow and levels of genetic contamination were investigated in a planted stand of *Acacia saligna* subsp. *saligna* via paternity analysis using microsatellite markers. High levels of outcrossing were detected within the stand (t_m = 0.98) and the average pollen dispersal distance was 37 m with the majority of progeny sired by paternal trees within a 50 m neighbourhood of the maternal tree. Genetic contamination from the natural background population of *A. saligna* subsp. *lindleyi* was detected in 14% of the progeny of *A. saligna* subsp. *saligna* and varied among maternal trees. Long distance inter-subspecific pollen dispersal was detected for distances of over 1500 m. The results provide information for use in the breeding and domestication program aimed at developing *A. saligna* as an agroforestry crop for the low rainfall areas of southern Australia.

Introduction

Tree species tend to be highly outcrossing and exhibit extensive levels of pollen mediated gene flow over large distances especially when populations are fragmented (Nason and Hamrick 1997; Dick 2001; White et al. 2002; Bacles et al. 2005; Byrne et al. 2007). Understanding the mating system and patterns of pollen dispersal in tree species is important for achieving conservation and natural resource management aims as well as for success in breeding programs for improvement and domestication.

In the context of breeding and domestication programs, knowledge of mating systems, patterns of pollen mediated gene flow and levels of genetic contamination are vital for the efficient management of production populations such as seed orchards (El-Kassaby 2000). Seed orchards are cultivated for the abundant production of easily harvestable seed from superior trees and are established by setting out clones or seedling progeny of trees selected for desired characteristics. Most tree seed orchards are open pollinated and aim to produce seed crops of high genetic value capturing an adequate amount of genetic diversity (Kang et al. 2001).

The genetic efficiency of seed orchards can be adversely affected by a number of factors including a high proportion of selfing events, departures from random mating due to factors such as unequal contribution of individuals to seed crops, and genetic contamination with pollen from trees located outside the orchard (Friedman and Adams 1985). To implement suitable orchard design and management options and achieve predicted genetic gains, it is useful to have knowledge of outcrossing rates, patterns of pollen dispersal, and maternal and paternal reproductive contributions. It is also important to determine the levels of long distance gene flow that may lead to genetic contamination from outside the orchard (Adams and Burczyk 2000).

Acacia saligna is native to southwestern Australia and has a long history of utilisation across Australia and worldwide. It is planted in many temperate and semi-arid countries for erosion control and sand dune stabilisation, and as a source of firewood and fodder for sheep and goats (Crompton 1992; Midgley and Turnbull 2003). The species is now a priority for further development as a short rotation phase or coppice agroforestry crop for the low rainfall agricultural areas of southern Australia (Olsen et

al. 2004; Hobbs et al. 2006). A breeding and domestication program for *A. saligna* commenced in 2004 when extensive germplasm collections were made and intensive field performance and progeny testing will select for improved growth rate, form, biomass production and feedstock characteristics (Olsen et al. 2004).

While the ecological characteristics of the *A. saligna* species complex are well described (Fox 1995), little is known about the mating system and patterns of gene flow via pollen dispersal that can be expected within and between populations. Mating systems and pollen dispersal parameters can be directly estimated through paternity analysis using genetic markers. Microsatellite markers or short sequence repeats (SSRs) are suitable for studies of pollen dispersal as they are highly polymorphic, codominant, considered to be selectively neutral, and can be amplified from small amounts of genomic DNA. As part of a larger effort in the breeding and domestication program, we developed and screened microsatellite markers in *A. saligna* and used them to investigate the mating system and pollen dispersal via paternity analysis for a planted stand of *A. saligna* subsp. *saligna.* The specific aims of the study were to determine (1) the level of outcrossing, (2) patterns of pollen dispersal, (3) relative paternal contributions to seed crops within the stand, and (4) the extent of genetic contamination via pollen dispersal from the background population of *A. saligna* subsp. *lindleyi*. Seed production areas have not been established for *A. saligna* and this data will be used to design efficient seed orchards as part of the breeding and domestication program.

Materials and Methods

Study species

Acacia saligna is a bushy shrub or tree that grows to 2-10 m at maturity, and lives for up to twenty years under favourable conditions (Fox 1995). It is a leguminous species that is allogamous and insect pollinated. Its natural distribution in southwestern Australia has a Mediterranean climate with annual rainfall of between 300 and 1200 mm mainly in the winter months. Trees bear hermaphroditic, many flowered, globular inflorescences in late winter to early spring (Maslin 2001). *Acacia saligna* has a widespread native range where it occupies a variety of habitats. Morphological and genetic studies have revealed that *A. saligna* is a species complex that exhibits a large degree of variation (Byrne and Millar 2004; George 2005) and consists of four main

variants to be formally described as subspecies (McDonald MW and Maslin BR ms). *Acacia saligna* subsp. *saligna* is native to the coastal areas around Perth and on the south coast, and shows the greatest biomass and floral fecundity of the four subspecies. This subspecies has been commonly used as an ornamental and in revegetation programs, and is likely to be the subspecies most commonly utilised for agroforestry. *Acacia saligna* subsp. *lindleyi* is the most widespread of the subspecies and occurs across the inland "wheat and sheep belt" area of Western Australia, which is the target area for revegetation.

Study site and sample collection

The study site consists of a planted stand of *A. saligna* subsp. *saligna* within the natural distribution of subsp. *lindleyi* and is located south east of Toodyay, Western Australia. The planted stand occupies an area of approximately 0.55 hectares (Figure 3.1) and occurs on a grassed paddock at a corner of the intersection of two roads. Tree spacing is uneven probably due to variable planting and survival. Remnant roadside trees of subsp. *lindleyi* occur at varying distances from the planted stand. The largest patch of remnant subsp. *lindleyi* trees occurs directly across a road at a distance of up to 330 m away from the *A. saligna* stand and other trees occur in two patches roughly 900 m and 1500 m away. All trees within the planted stand and 29 selected trees of subsp. *lindleyi* were mapped using a differential global positioning satellite system.

Mature phyllode material was collected from all 107 trees within the stand of subsp. *saligna* and from 29 selected trees of subsp. *lindleyi* in December 2004. Open pollinated seed was sampled from 10 trees of *A. saligna* subsp. *saligna* chosen at random from within the stand in December 2005. Seed was sterilised in 1.0% sodium hypochlorite solution for one hour and rinsed thoroughly in sterile water. Boiling water was poured over the seeds and they were left to imbibe overnight. No seed germinated for one of the maternal trees so seed crops were analysed for nine maternal trees.

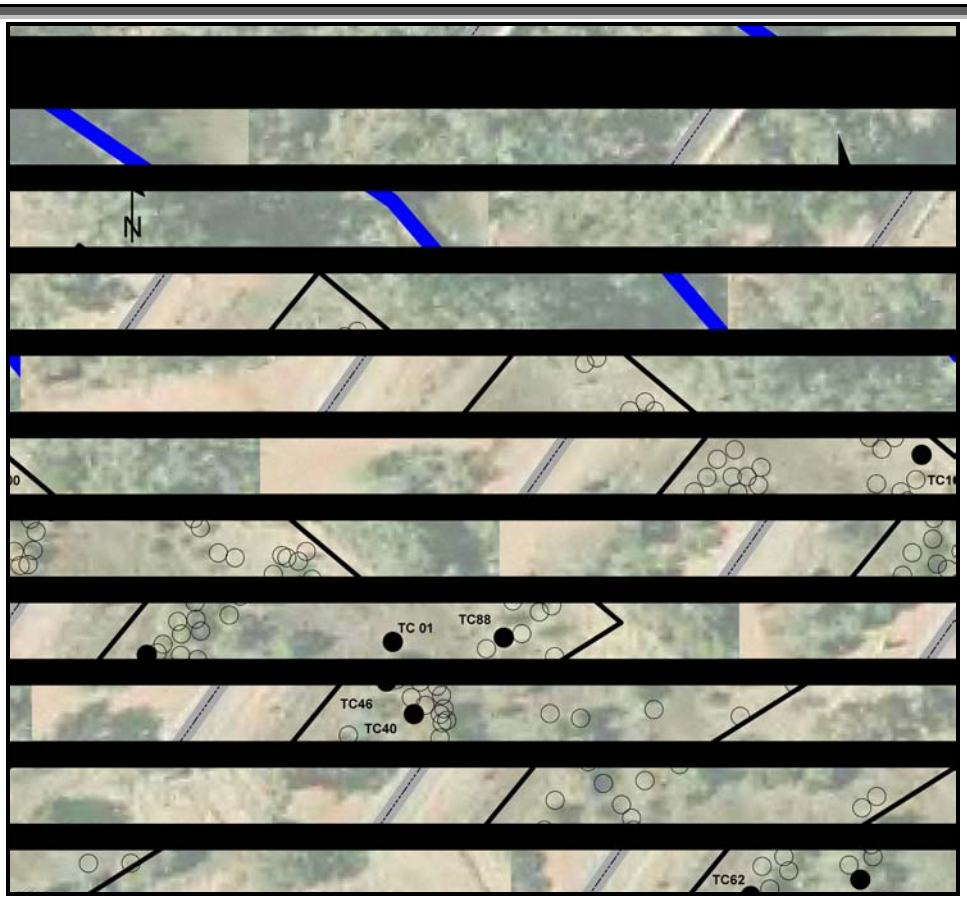


Figure 3.1 Map of the study site near Toodyay, Western Australia showing the planted stand comprised of 107 *A. saligna* subsp. *saligna* trees (open circles) and individual locations of sampled maternal trees (closed circles). Broken grey lines are roadways and the blue line is the Avon River.

Microsatellite marker development

A microsatellite library was developed using a hybridisation selection method. Genomic DNA was extracted from adult phyllodes using a modified cetyltrimethyl ammonium bromide (CTAB) extraction method (Byrne et al. 1993) with the addition of 0.1 M sodium sulfite to the extraction and wash buffers (Byrne et al. 2001). Genomic DNA (10 µg) was digested with *Sau*3A and ligated into 2 µg of pUC18 plasmid vector then transformed into competent *Escherichia coli* DH5α cells (Gibco) according to the manufacturer's protocol. The resultant colonies were screened by colony lift hybridisation onto Hybond-N+ membranes using γ-^{32} P – dATP (PerkinElmer Life Sciences) end labelled dinucleotides (AC/AG). Hybridisation was carried out with equal concentration (100 ng/30 ml) of the nucleotide probes in 6 X SSPE, 5 x Denhardts, 1% SDS at 65^{o}C overnight. Following three post hybridisation washes in 0.5 x SSC, 0.1% SDS, the membranes were exposed to radiography film. Positive

colonies were picked and gridded onto new membranes then re-screened by hybridisation with the probes.

Positive colonies were grown in LB media overnight at 37°C. Plasmid preparations were carried out with UltraClean Mini Plasmid Prep Kit (MoBio Laboratories) kit following the manufacturer's instructions. Plasmid inserts were sequenced using BigDye Terminator chemistry and 12 of 16 clones (75%) contained a microsatellite sequence of eight or more repeats. Sequences flanking the microsatellite repeat motifs were analysed and compatible primer sequences were identified for nine sequences using PRIMER 3 (Rozen and Skaletsky 2000). Primer pairs were tested for mispriming and primer dimers with AMPLIFY 1.2 (Engels 1993).

Microsatellite loci were amplified in a total volume of 15 µl per reaction, containing 30 ng template DNA, 50 mM KCl, 20 mM Tris HCl pH 8.4, 0.2 mM each dNTP, 0.3 µM of each primer, 0.5 units of Taq DNA polymerase and variable $MgCl_2$ concentration depending on the locus (see Table 3.1). Amplification conditions used either Protocol 1: 96°C for 2 min, 30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C followed by 5 min at 72°C; or Protocol 2: 94°C for 2 min, 30 cycles of 30 s at 94°C, 30 s at 65°C with a step down of 0.3°C per cycle to 56.3°C, 5 s at 72°C, 3 cycles of 30 s at 94°C, 30 s at 55°C, 5 s at 72°C followed by 5 min at 72°C. All reactions were completed in a Touchdown Thermal Cycler (Hybaid Ltd). The amplification products were subjected to electrophoresis on 8% polyacrylamide gels and stained with ethidium bromide. Suitable primers were selected based on the production of amplification products that could be interpreted as single loci

In addition, microsatellite markers developed for *A. mangium* (Butcher et al. 2000) and the hybrid *A. mangium* x *A. auriculiformis* (Ng et al. 2005) were tested for cross amplification in *A. saligna*. A range of amplification conditions was tested and loci were considered suitable for use in *A. saligna* when sharp bands were produced that showed polymorphism that could be interpreted as a single locus.

Table 3.1 Details of five microsatellite loci used to genotype individuals of *Acacia saligna*.

Locus	Genbank accession number	Primer sequence (5'-3') [a]	Repeat motif [a]	PCR protocol	$MgCl_2$ conc (mM)
As029	EF194131	F: CTTCCTTTGCCACCCTTTTG R: GGTTTGGAACATTGTGAAGTCG	$(AC)_{11}$	2	1.5
As137	EF194132	F: GAGGTAATATTTTGAATTCCTTGAAC R: GGTGTATACCTCTTTCCTGTGG	$(AT)_9(GT)_{15}$	1	1.5
Am012	NA	F: CCAGGAAACCACATAAACAACC R: AGGAATTGGGGTTTCTCAGG	$(AC)_8$	1	2.0
Am030	NA	F: TGAGTCGATCGCTTAGCTTG R: TCCCGTTATTATGCCAAAGTG	$(TC)_{15}(AC)_7$-$(AC)_{10}AG$	1	1.5
Am367	NA	F: CGCAACTCCATCTGATTTACTG R: TTATGTTGGGTTAATACGCTAACTG	$(A)_7G(A)_6GG(A)_{14}(CA)_{14}$	1	1.5

Primers developed for *A. saligna* designated by As; primers developed for *A. mangium* designated by Am.

[a] Repeat motif and primer sequence for *A. mangium* primers taken from Butcher et al. 2000.

DNA extraction and genotyping

Genomic DNA was extracted from phyllodes of the adult trees as described above. DNA was extracted from planted seedlings that had grown to a height of approximately 5 cm, using a modified Doyle and Doyle procedure (Doyle and Doyle 1990). Genotypes of all *A. saligna* subsp. *saligna* trees in the planted stand, progeny arrays for nine families consisting of 11-22 seedlings (186 in total), and 29 *A. saligna* subspecies *lindleyi* trees were determined for five microsatellite loci as described above except that the size of PCR products was determined by automated fluorescent scanning detection using an Applied Biosystems 3730 DNA Analyser (Applied Biosystems, Foster City CA, USA). Random sets of samples were rerun to check for repeatability of genotyping and segregation of maternal alleles in the progeny was checked.

Data analysis

Genetic diversity parameters

Genetic diversity parameters were computed for the adult trees in the study stand, for the progeny alone, and for the adults and progeny combined using the CERVUS 2.0 program (Marshall et al. 1998). Differences between observed and expected heterozygosity of adults and progeny combined and as separate cohorts were assessed using the chi-squared (χ^2) test.

In order to quantify the genetic heterogeneity of pollen pools among maternal trees of *A. saligna* subsp. *saligna* analysis of molecular variance (AMOVA) (Excoffier et al. 1992) based on pollen haplotypes was performed using TWOGENER (Smouse et al. 2001). This provides a 'global' estimate of spatial genetic variation of pollen pools over all maternal trees, Φ_{ft}. Pollen haplotypes were derived by subtracting maternal haplotype components from the progeny genotypes. Homogeneity of gene frequencies in the progeny of maternal trees was tested using chi-squared and likelihood ratio (G^2) tests implemented in POPGENE (Yeh et al. 1997).

Mating system

Estimates of mating system parameters were produced using the multilocus estimator program MLTR version 3.0 (Ritland 2002). Analysis used the Expectation-Maximisation (EM) method for determining maternal and pollen allele frequencies and the Newton Raphson method for joint maximum likelihood estimation of the multilocus outcrossing rate (t_m) and single locus outcrossing estimate averaged over all loci (t_s). Estimates of biparental inbreeding (t_m-t_s), correlation of paternity (r_p) and the correlation of *t* among loci (r_s) were also obtained. Standard errors were computed based on 500 bootstraps. The correlation of paternity was translated to provide estimates of the effective number of pollen donors using the equation $N_{ep} = 1/r_p$ (Ritland 1989). The standard error was used to determine whether mating parameters were significantly lower than one or greater than zero. Pollen and ovule frequencies were estimated for each locus using the EM method.

Paternity assignment

Maximum likelihood-based paternity assignment was conducted using CERVUS 2.0 (Marshall et al. 1998). The total size of the candidate male breeding population used in paternity assignment included the 107 trees of *A. saligna* subsp. *saligna* in the study stand and an estimated 50 candidate male parents of *A. saligna* subsp. *lindleyi*. Though more than 50 trees of subsp. *lindleyi* that may have been candidate male parents were present, the majority were not at reproductive maturity and had limited flowering. Cryptic gene flow (Devlin and Ellstrand 1990) was estimated using FAMOZ software (Gerber et al. 2003) for a simulated population developed using data for the mother trees and progeny of *A. saligna* subsp. *saligna*. Parameters for estimates of Type I (α)

and Type II (β) errors included 500 simulated progeny, 20 mothers, and an error rate of 0.008.

Spatial Analysis

Pollen dispersal patterns within the planted stand were assessed by spatial analysis. The geographic distribution patterns of pollen donors were plotted for nine maternal trees in order to visually assess any bias in direction for pollen dispersal. Average pollen dispersal distances were assessed using the Kruskall-Wallace test for non-normal data to determine whether pollination distances varied between maternal trees. Paternal contributions of trees in the *A. saligna* subsp. *saligna* stand were plotted as a function of the number of progeny fathered. Male mating success as a function of distance from maternal trees was analysed by subdividing the neighbourhood of each mother tree into increasing classes of 10 m increments and plotting the frequency distribution of all successful pollination events for paternal trees in each distance class.

A dispersal curve was fitted to the observed pollen dispersal distances over all maternal trees within the stand where f (x) is the contribution (in relative frequency) of paternal trees as a function of distance to maternal trees. A number of models suitable for describing the pollen dispersal kernel were tested including normal, exponential, exponential power, Weibull, geometric and 2Dt curves.

To take into account the differing number of potential male parents in each distance class for each maternal tree, the proportion of observed male parents (ratio of the number of observed successful male parents located in the distance class over the total number of observed successful male parents over all classes within the stand) was compared with the proportion of potential male parents in each distance class (ratio of the number of possible male parents in the class over the total number of possible male parents over all classes within the stand). In this analysis, observed successful male parents were counted no more than once (even when male parents were assigned to multiple progeny of a single maternal tree) since the probability of siring multiple progeny was considered equal among pollen donors of subsp. *saligna*. All trees of subsp. *saligna* were fecund showing high levels of floral production. Significant deviation between the number of potential and observed male parents for near (<50 m)

and far (>50 m) distances, averaged over all maternal trees was tested using a chi-squared contingency test.

Results

Microsatellite marker development and utility

Only two of the nine primers developed from the constructed microsatellite library produced amplifiable products interpretable as single loci. The remaining primers appeared to amplify multiple loci, which could not be resolved even under high stringency conditions. Of the 25 loci tested for cross species amplification, three from *A. mangium*, Am012 Am030 and Am367, showed clear amplification in *A. saligna*. No primers developed for the *A. mangium* x *A. auriculiformis* hybrid produced suitable amplification that could be interpreted as single loci in *A. saligna*.

Table 3.2 Utility of five microsatellite loci for *Acacia saligna* subsp. *saligna* at the study site.

Locus	*A*	H_o	H_e	PIC	Excl (2)	Null
As029	6.0	0.392	0.388	0.358	0.208	-0.0192
As137	6.0	0.323	0.308	0.294	0.170	-0.0365
Am012	10.0	0.747	0.779	0.401	0.581	0.0221
Am030	9.0	0.345	0.416	0.391	0.238	0.1027
Am367	16.0	0.783	0.890	0.878	0.775	0.0626
Mean	9.4	0.518	0.556	0.534	0.953*	0.0263

Results are presented for parental and progeny cohorts combined.

A: number of alleles, H_o: observed heterozygosity, H_e: expected heterozygosity, PIC: polymorphic information content, Excl (2): power of exclusion for the second parent, Null: estimated frequency of null alleles.

*The total probability of exclusion for the second parent over all five loci is given.

The five microsatellite loci were highly variable in *A. saligna*. The total number of alleles for the five loci was 47, and the number of alleles per locus (*A*) ranged from six to sixteen and averaged 9.40 (Table 3.2). Observed heterozygosity (H_o) ranged from 0.323 to 0.783 and averaged 0.518. No significant differences were found in observed or expected heterozygosity for parents and progeny combined (χ^2 = 7.65, d.f. = 4, p-value = 0.105) or for parents (χ^2 = 0.0105, d.f. = 4, p-value = 0.99) and progeny (χ^2 = 0.054, d.f. = 4, p-value = 0.99) analysed as separate cohorts. Null allele frequency

averaged 0.0263 for trees of subsp. *saligna*. As mismatches generated by null alleles are treated as typing errors in paternity assignment, null alleles with low frequencies do not have a significant effect. The polymorphic information content (PIC) of the loci was high and the total exclusion probability over the five loci was 0.953 for the second parent.

Table 3.3 Details of outcrossing rate, pollen dispersal and gene flow estimates obtained via paternity analysis for nine maternal trees of *Acacia saligna* subsp. *saligna*

Maternal Tree	n	n_p	t	N_e	δ (m)	< 50 m	> 50 m	% genetic contam-ination
TC001	22	20	1.0	12	31 (15.6)	18	2	9
TC040	22	18	1.0	17	37 (15.6)	13	5	27
TC046	22	22	1.0	15	29 (9.9)	22	0	0
TC053	22	19	1.0	13	24 (19.6)	13	6	32
TC062	22	21	0.95	17	36 (24.4)	14	7	22
TC079	22	22	1.0	14	40 (25.1)	13	9	14
TC082	21	20	1.0	19	38 (18.7)	14	6	5
TC088	11	10	0.90	9	50 (31.5)	4	6	20
TC100	22	22	0.95	12	44 (35.0)	11	11	0
Mean	20.66	19.33	0.98 (0.03)	14.22	37 (2.3)	122*	52*	14

Values in parenthesis are standard errors.

n: number of progeny analysed, n_p: number of progeny assigned a father, t: outcrossing rate, N_e: number of paternal trees contributing to progeny, δ: average observed dispersal distance within the *saligna* stand (m), < 50 m: number of progeny for which pollen dispersal distance was less than 50 m, > 50 m: number of progeny for which pollen dispersal distance was greater than 50 m, % genetic contamination: percentage of progeny sired by trees outside the stand.

* Totals are given.

Mating system

Multilocus outcrossing within the study stand was high (t_m = 0.98) and showed little variation among maternal trees (from 0.90 to 1.0, Table 3.3). This indicates a mixed mating system with small amounts of inbreeding. Correlation of selfing within progeny arrays, an indication of the fraction of inbreeding due to uniparental inbreeding or true selfing, was low (r_s = 0.125). The correlation of paternity is a measure of the probability that pollination events produced full sib relatives and was high (r_p = 0.234). This estimate then provided a low value for the effective number of pollen donors (N_{ep} = 4.27 donors per maternal tree).

Paternity Assignment

Using the five loci, 93.5% of analysed progeny were assigned a most likely father from either within the subsp. *saligna* stand or from the sampled trees in the background population of subsp. *lindleyi*. The majority of progeny (86%) were sired by pollen from within the stand of subsp. *saligna*. Fourteen progeny (7.5%) were assigned a father from the sampled subsp. *lindleyi* trees. The paternity of twelve progeny (6.5%) was unassigned and they were assumed to be sired by unsampled trees of the background population of subsp. *lindleyi*. The rate of pollen contamination into the subsp. *saligna* stand is the percentage of progeny whose paternity was not assigned to subsp. *saligna* trees and was 14%. Three selfing events were detected, one each for maternal trees TC062, TC088 and TC100. Outcrossing rates determined by paternity assignment ranged from 0.90 to 1.0 for maternal trees and averaged 0.98. The estimate of outcrossing obtained via direct paternity assignment was the same as that determined by the mating system assessment using the mixed mating model.

Over half (59.8%) of the subsp. *saligna* trees within the study stand contributed to the pollen pool, siring at least one offspring (Figure 3.2). One paternal tree (TC053) sired nine offspring, representing 5.2% of the progeny analysed. There were 25 instances of correlated paternity detected within the stand with up to six progeny of a single maternal tree sired by the same father. The average number of progeny sired by paternal trees within the stand was 2.3. Half of the paternal trees that contributed to the effective pollen pool sired only one progeny.

Spatial Analysis

Analysis of patterns of pollination within the planted stand showed individual observed pollen dispersal distances ranging from 5 to 96 m. The average pollination distance for each individual mother tree ranged from 24 m to 50 m and was not significantly different among mother trees ($\chi^2 = 12.50$, df = 8, p = 0.130). The average dispersal distance was 37 m and was similar to the average geographic distance between the maternal trees and all other trees of 40 m. Two pollination events were detected where pollen travelled a distance of 96 m. This is the maximum distance possible at this site and was observed for mother tree TC100, which is situated at the north eastern edge of the stand and hence had the opportunity for such long distance pollination events.

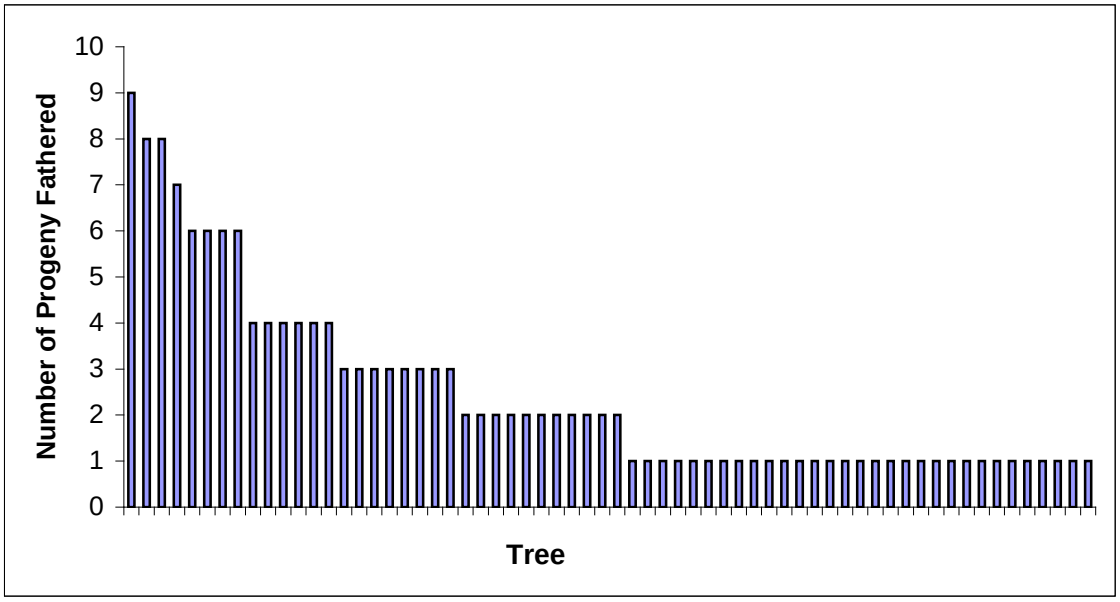
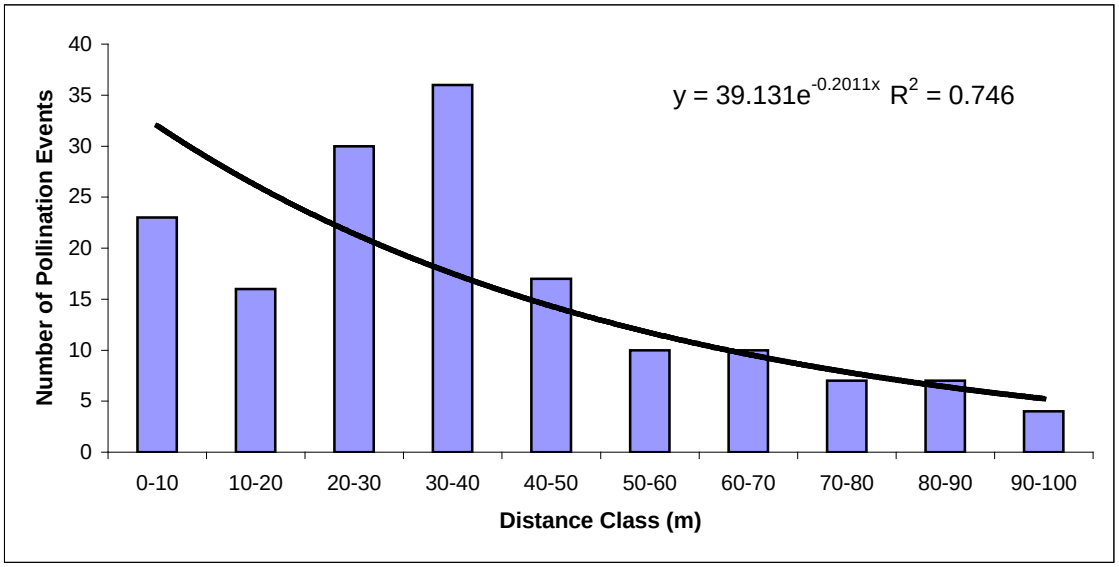


Figure 3.2 Male mating success of the 64 trees in the planted stand of *Acacia saligna* subsp. *saligna* that sired progeny assayed from nine maternal trees.

Analysis of pollination events within the stand plotted over dispersal distance classes showed that for most maternal trees, the majority of pollination events were due to pollen from paternal trees within a 50 m neighbourhood (Figure 3.3). For maternal tree TC100, pollination events from near (< 50 m) and far (> 50 m) paternal trees were equal and for one maternal tree, TC088, the majority of pollen came from paternal trees greater than 50 m away. Of the dispersal curves tested, the negative exponential model gave the best fit and explained the highest percentage of observed mating events (R^2 = 0.746).

Analysis of dispersal distance class in Figure 3.3 does not take into account the variable number of potential pollen donors in each distance class due to unequal spacing and the position of the maternal trees. To determine any bias in pollination distances the distribution of the potential and observed male parents was plotted as a function of distance to maternal trees (Figure 3.4). The deviation between the potential and observed parents depicts the departure from random mating caused by the effect of distance between parental trees on male mating success. The number of observed compared to potential male parents shows a significant excess of near (< 50 m) matings (χ^2 = 10.47, df = 1, p = 0.001) when averaged over the nine maternal trees and the majority of successful paternal trees (74%) were located at distances of < 50 m from



maternal trees.

Figure 3.3 Distribution of pollen dispersal distance classes in progeny assayed from nine maternal trees of a stand of *Acacia saligna* subsp. *saligna*. The line is the exponential curve fitted to the mating frequencies averaged over maternal trees.

Although it was not empirically tested, pollination events detected by parentage assignment within the stand indicate a random dispersal pattern. No strong bias in direction of pollen travel due to prevailing winds was observed and the distribution of pollen donors for each maternal tree were independent of their position in the stand (data not shown). However, a moderate level of genetic heterogeneity among pollen pools of maternal trees was evident in a global Φ_{ft} of 0.1108. Heterogeneity in pollen clouds across maternal trees was also detected at each locus using tests for homogeneity ($p < 0.01$) (Table 3.4). G^2 values are reported as they have a more accurate approximation to the log likelihood ratios of the theoretical χ^2 distribution.

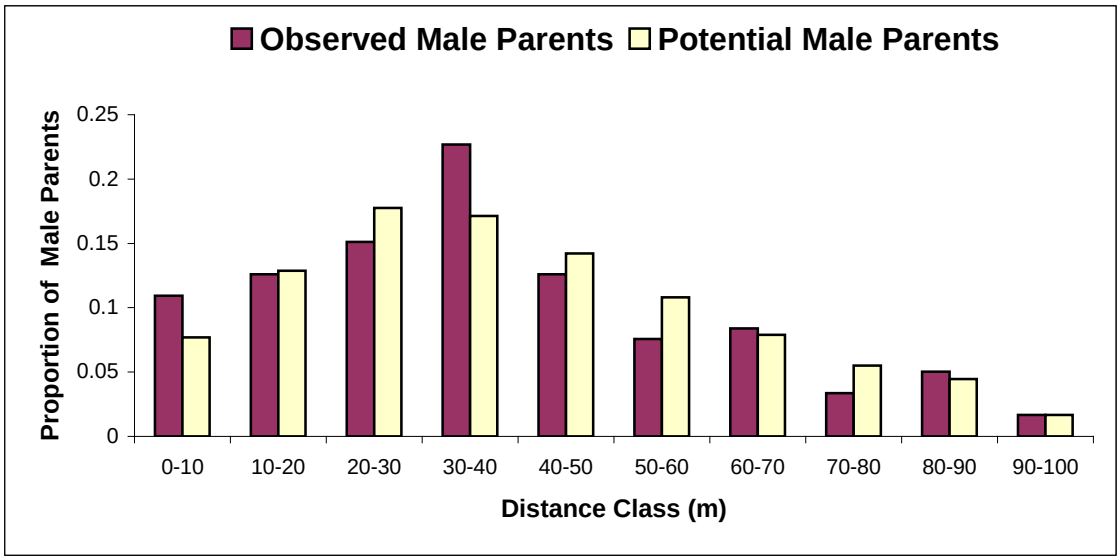


Figure 3.4 Comparison of the potential (light bars) and observed (dark bars) pollinating male parent distribution as a function of distance to the female parent. The potential distribution comprised all 107 trees within the planted stand assuming random pollen dispersal.

Genetic Contamination

Genetic contamination via pollen from *A. saligna* subsp. *lindleyi* trees into the planted stand of subsp. *saligna* was 14%. The level of genetic contamination can be adjusted for Type I errors, or the failure to assign a father to a progeny when the true father has been genotyped, and for Type II errors that occur when a progeny is assigned a father from the sampled trees when its true father lies in the unsampled background population. Allowing for these errors, the number of progeny assigned an *A. saligna* subsp. *saligna* father that were actually sired by ungenotyped subsp. *lindleyi* trees may have been underestimated by 11.5%.

Genetic contamination rates varied among maternal trees within the stand and ranged from 0 to 32% (Table 3.3). Of the 29 trees of *A. saligna* subsp. *lindleyi* that were sampled, 10 were assigned as fathers of one or more progeny in the sampled seed crops from the planted stand. Inter-subspecific pollination events due to pollen sources from the background population of subsp. *lindleyi* occurred over a range of distances from

Table 3.4 Pollen allele frequencies (only two most common alleles are shown) and significance for G^2 tests of homogeneity between pollen clouds for nine maternal trees of *A. saligna* subsp. *saligna*.

Maternal Tree	Locus									
	As029		As137		Am012		Am030		Am367	
	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
TC001	D	0.8363	E	0.7500	D	0.2500	A	0.0526	D	0.3000
	E	0.0455	F	0.1500	E	0.3000	B	0.9474	M	0.3000
TC040	A	0.0273	E	0.8182	C	0.4211	B	0.8571	D	0.1818
	D	0.5455	F	0.0909	D	0.2105	C	0.0952	J	0.1818
TC046	D	0.4091	A	0.1818	D	0.3810	A	0.2857	C	0.6364
	E	0.5455	F	0.8182	E	0.2381	D	0.4286	M	0.1818
TC053	A	0.2273	D	0.1364	D	0.5000	B	0.8095	C	0.1111
	D	0.5909	E	0.7727	E	0.3000	E	0.0952	D	0.3889
TC062	A	0.1364	B	0.1818	B	0.2105	A	0.2381	E	0.2500
	D	0.7273	E	0.5909	D	0.4211	B	0.6190	I	0.2500
TC079	D	0.5000	B	0.2273	D	0.3810	A	0.1364	I	0.1818
	E	0.2727	E	0.6818	F	0.2857	B	0.6364	L	0.4091
TC082	D	0.6190	A	0.1429	D	0.4500	A	0.0952	L	0.3333
	E	0.3810	E	0.8571	H	0.2000	B	0.8095	N	0.2381
TC088	A	0.4545	A	0.0909	B	0.2222	B	1.0	D	0.1818
	D	0.5455	E	0.9091	C	0.3333	-	-	M	0.4545
TC100	B	0.0455	B	0.0455	C	0.4286	B	1.0	J	0.1429
	D	0.9091	E	0.9545	E	0.3333	-	-	L	0.5238
G^2	64.54		64.68		108.28		92.85		264.98	
d.f.	32		40		56		48		104	
p value	0.00057		0.00802		0.00003		0.00011		0.00000	

d.f.: degrees of freedom.

5 m to 1566 m with an average pollen dispersal distance of 666 m. The level of pollen contamination from trees of subsp. *lindleyi* that were close to the planted stand (< 130 m away) was similar (57% of all contamination) to the level of genetic contamination that occurred via pollen from the two outlying patches of trees of subsp. *lindleyi* (43% of all contamination).

Discussion

The design and genetic efficiency of seed orchards are affected by biological aspects of taxa including patterns of pollen mediated gene flow, levels of outcrossing and random mating, and genetic contamination from outside the orchard. This study assessed these factors for a planted stand of *A. saligna* subsp. *saligna* and found high outcrossing with generally random mating and pollen dispersal within the stand, and a low level of genetic contamination from nearby natural remnant populations of subsp. *lindleyi*.

The multilocus outcrossing rate within the study stand was high and varied little for maternal trees. The high level of outcrossing is similar to levels found in a previous study using allozyme markers for several natural and one planted population of *A. saligna* (George 2005). High outcrossing rates (t_m >0.9) have also been found for other *Acacia* species including *A. auriculiformis* A. Cunn. Ex Benth. *A. crassicarpa* A. Cunn. Ex Benth. (Moran et al. 1989b), plantations of the important tannin-producing species *A. mearnsii* (Kenrick and Knox 1989b) and some populations of *A. mangium* Willd. (Butcher et al. 2000). The outcrossing rate for *A. saligna* is much higher than that found in two other species that are native to Western Australia, *A. sciophanes* and *A. anfractuosa* (Coates et al. 2006).

The pattern of mating within the stand was essentially random. Although a significant excess of matings was detected at distances of less than 50 m, the average distance of pollen dispersal was very similar to the average distance between maternal trees and all potential pollen donors and pollen dispersal was observed across the maximum distance of the stand. There was no evidence of directionality in pollen dispersal and no evidence of a majority of nearest neighbour mating. There was evidence of heterogeneity in the genetic composition of pollen clouds experienced by maternal trees indicating that they were not sampling a single pollen cloud. However, this may be due

to the high genetic diversity of the stand and high heterozygosity in the trees due to a diverse source of germplasm. Such high heterozygosity would lead to patchy distributions of localised allele frequencies in the pollen pool and would be expected to result in heterogeneity in paternal contributions when assessed for a subset of progeny. The levels of heterogeneity in pollen clouds found for the study stand therefore also do not necessarily imply that maternal trees are sampling only highly localised or restricted pollen pools.

Although there was essentially a pattern of random mating within the stand, there was some evidence of correlated paternity. Correlated paternity is generally indicative of genetic structuring within natural populations, but the values found here are more likely to be due to the nature of *Acacia* floral biology. In *A. saligna*, flowers are grouped into complex inflorescences and pollen from the same paternal tree may regularly fertilise more than one flower within a cluster. In addition, pollen grains are compound in nature occurring as polyads of 16 grains, and there is correlation between the number of ovules within a flower and the number of pollen grains per polyad across *Acacia* species (Kenrick and Knox 1982). Hence, one polyad may be sufficient to sire all progeny within a pod. These aspects of floral biology have been shown to produce high levels of correlated paternity in another *Acacia* species (Muona et al. 1991). Correlated paternity due to this aspect of the floral morphology is not related to variable fecundity in trees of subsp. *saligna* and all trees have equal probability of contributing to the correlated paternity of the seed crop, thus, the potential for random mating is maintained.

Random mating within seed orchards will be promoted by random pollen dispersal patterns, synchronised floral phenologies, equal compatibility of individuals and equality in individual reproductive success. The observations of high outcrossing and random pollen dispersal found here for the subsp. *saligna* stand indicate that conditions approaching panmixia may be expected for seed orchards established in this species. Thus, seed orchards are an effective means of producing high value seed for deployment of *A. saligna* in agroforestry plantings.

One factor that may require active management in seed orchards of *A. saligna* subsp. *saligna* is the level of genetic contamination from outside the orchards. Inter-subspecific pollen dispersal from subsp. *lindleyi* trees in the nearby natural population

was detected over large dispersal distances of up to 1566 m. In the case of seed orchards, pollen dispersal from divergent populations should be minimised in order to maintain genetic integrity in subsequent seed crops and prevent the dilution of advanced breeding genotypes (Friedman and Adams 1985; Adams and Burczyk 2000). The magnitude of genetic contamination is difficult to predict for given taxa but even low levels may be detrimental to the efficiency of seed orchards. Levels of genetic contamination are affected by the relative amounts of pollen produced in pollen source and sink populations, and the distance between populations (Ellstrand 1992). The planted stand of subsp. *saligna* studied here (sink) is smaller than most commercial seed orchards and large numbers of subsp. *lindleyi* trees (source) are located nearby. However, the majority of subsp. *lindleyi* trees were not at reproductive maturity at the time of sampling and floral fecundity of subsp. *lindleyi* was much lower than that of subsp. *saligna*. Hence, the total amount of pollen produced by subsp. *lindleyi* trees was probably less than that of the subsp. *saligna* stand. Disparity in overall pollen production may be responsible for the relatively low level of genetic contamination found here despite the proximity of subsp. *lindleyi* trees to the subsp. *saligna* stand. However, the large distance over which contamination occurred indicates that distance itself is not a strong barrier to pollen dispersal in this species.

The level of genetic contamination found for *A. saligna* indicates that management options that reduce the amount of genetic contamination into seed orchards will be required. The long distance intraspecific gene flow found in this study gives an indication that minimum isolation distances required would be greater than 1500 m. In addition, subsp. *lindleyi* flowers slightly later than the peak flowering time of subsp. *saligna* therefore selective thinning of late flowering individuals may reduce the amount of genetic contamination within seed orchards as these trees would be more susceptible to pollination from subsp. *lindleyi*.

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Chapter Four
Long distance inter-subspecific gene flow into remnant *Acacia saligna* (Mimosaceae) from a planted stand.

Statement of Contribution

M. A. MILLAR

Conducted the site selection, experimental design, all fieldwork, all laboratory work, all data and results analysis, wrote the final paper and acted as corresponding author

Signed Date

M. BYRNE

Provided assistance with theoretical conceptualisation, experimental design and writing the final paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

I. NUBERG

Reviewed and provided comment on final drafts of the paper

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

M. SEDGLEY

Reviewed and provided comment on final drafts of the paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

Chapter Four

Long distance inter-subspecific gene flow into remnant *Acacia saligna* (Mimosaceae) from a planted stand.

Running Title: Inter-subspecific gene flow in **Acacia saligna**

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Key words: pollen-mediated gene flow, paternity analysis, *Acacia saligna*

Abstract

It is essential to understand patterns of gene flow in remnant plant populations occupying highly disturbed landscapes in order to provide sustainable management options that ensure long-term population persistence. Paternity analysis was used to assess pollen-mediated gene flow between a planted stand of *Acacia saligna* subsp. *saligna* (ms) and small remnant patches of indigenous *Acacia saligna* subs. *lindleyi* (ms) in southern Western Australia. High levels of inter-subspecific pollen immigration (32%) were detected in remnant roadside populations of subsp. *lindleyi.* Lower levels of inter-subspecific pollen immigration were detected in a planted stand of the more florally fecund subsp. *saligna* (14%). The majority of intra-subspecific pollen immigration into remnant patches of subsp. *lindleyi* occurred at distances of less than 300 m, however a level of long distance pollen immigration over 1500 m was detected. Levels of intrapopulation mating decreased as patches of subsp. *lindleyi* became smaller and more spatially isolated. The results indicate that remnant populations of woody perennial species may be exposed to substantial amounts of inter-subspecific pollen-mediated gene flow when large populations of genetically divergent yet interfertile taxa are planted nearby small remnant patches of a less florally fecund congener. Until hybrid fitness and the likely impacts of hybrid progeny on the persistence of remnant populations of subsp. *lindleyi* are assessed, management practices that reduce the amount of pollen immigration into remnant populations from genetically divergent introduced populations are recommended.

Introduction

Natural vegetation occupying highly anthropogenically-disturbed landscapes is often fragmented in comparison to pre-disturbance conditions, resulting in decreased population size and density, and increased isolation through distance or other barriers (Kwak et al. 1998). Population genetic theory predicts that levels of gene flow will be reduced for such fragmented and isolated populations (Slatkin 1987; Young et al. 1996) and this may increase the impacts of small population processes, such as inbreeding and genetic drift (Slatkin 1987; Lande 1988; Young et al. 1996). A number of studies have assessed the genetic consequences of altered patterns of pollen-mediated gene flow in fragmented natural populations following anthropogenic disturbance such as land clearing (Young et al. 1996; Nason and Hamrick 1997; White et al. 2002; Lowe et al. 2005) and a growing literature shows that pollen-mediated gene flow in most fragmented tree species does not follow the predictions of classical genetic theory. Tree species in fragmented populations often show extensive levels of long distance pollen-mediated gene flow, which may have actually increased following habitat fragmentation, providing interconnectivity between populations (Nason and Hamrick 1997; White et al. 2002; Dick et al. 2003; Byrne et al. 2007).

While increased pollen-mediated gene flow among fragmented populations in a natural system will have positive effects in reducing inbreeding and drift, unrestricted pollen-mediated gene flow between genetically divergent populations may be undesirable and have negative consequences for the long-term persistence of natural populations. Extensive pollen-mediated gene flow may result in the production of hybrid progeny and the establishment of hybrid populations (Arnold 1992; Ellstrand 1992) and pollen swamping may be detrimental to the viability of natural populations regardless of whether viable hybrid progeny are produced (Young et al. 1996; Wolf et al. 2001). While the long term impacts of extensive pollen immigration and hybridisation are highly unpredictable for given taxa they do depend on the level of genetic differentiation between populations and the nature of the evolutionary process generating the divergence (Ellstrand and Ellam 1993).

To date, few studies have assessed the genetic consequences of further anthropogenic disturbance in fragmented landscapes that is likely to result in increased levels of

pollen-mediated gene flow into fragmented populations. The likelihood and possible impacts of this scenario are receiving increased attention due to the threat of pollen-mediated gene flow and hybridisation between natural populations and introduced transgenic crops (Raybould and Gray 1994; James et al. 1998; Raybould 1999; Hails 2000; Armstrong et al. 2005). Pollen-mediated gene flow may occur when genetically divergent yet interfertile congeners are introduced into the range of native taxa, such as with intentional planting for agriculture, agroforestry or revegetation programs, or through the unintentional spread of invasive species. In such cases, genetic divergence between natural and introduced populations may have arisen through the use of non-local subspecies or provenance material, or selection pressure applied in breeding and domestication programs (Byrne and Millar 2004).

Across southern Australia, the majority of the once extensive woody perennial vegetation has been cleared for agriculture and the remaining native vegetation is largely composed of small, highly fragmented populations often relegated to roadside remnants (Glanznig 1995). There is an urgent need to revegetate this landscape with native perennial species in order to mitigate the effects of dryland salinity. To this end, native species are being developed for use in revegetation via agroforestry. There are significant advantages in using native species since they should be adapted to the harsh environment and nutrient deficient soils of southern Australia, have less risk of becoming economic and environmental weeds, and provide ready access to large genetic resources for development. However, large-scale plantings of certain native taxa suitable for agroforestry may pose a risk to native congeners via pollen immigration. The extensive pollen-mediated gene flow identified in studies of tree species in fragmented landscapes suggests that pollen-mediated gene flow may also be extensive in the agricultural landscapes of southern Australia and the potential for pollen mediated ‘genetic contamination’ in remnant populations should be evaluated within a risk management framework (Ellstrand 1992; Potts et al. 2003).

This study was conducted to provide information on the levels and patterns of inter-subspecific gene flow for a priority species under development for agroforestry in southern Australia. *Acacia saligna* (Labill.) H.L.Wendl. has been identified as the most promising species for revegetation in southern Australia as it is fast growing, produces large amounts of biomass with multiple uses, and can be grown as a short rotation phase

or coppice crop. The species has a naturally widespread distribution across southern Western Australia and is naturalised in other areas in south-eastern Australia (Maslin 2001). *Acacia saligna* is currently recognised as a species complex, comprised of four main entities to be raised to subspecies (McDonald and Maslin in prep). The four proposed subspecies display great morphological variation and have been shown to be highly genetically divergent using both RFLP (George et al. 2006) and microsatellite genetic data (Millar et al. 2008c).

As for most acacias, *A. saligna* has a highly outcrossed mating system (George 2005; Millar 2008a). Preliminary evidence suggests that the peak flowing times of the subspecies differ but overlap is observed in floral phenology (George 2005). The subspecies are also suitably interfertile for hybridisation to occur (George et al. 2006; Millar et al. 2008a). Because of the high level of genetic differentiation between the subspecies, it is likely that germplasm used for agroforestry will be genetically divergent from natural populations in the target area for revegetation. The subspecies with the greatest biomass production does not naturally occur in the area targeted for revegetation, the agricultural region that encompasses the distribution of a different subspecies. In addition, the subspecies likely to be used for agroforestry has much greater floral fecundity than that of the subspecies native to the agricultural region, thus potential contaminant pollen pools will be large in comparison to the smaller pollen pools of remnant populations. We expect that levels of pollen immigration from planted stands into natural populations of *A. saligna* will be extensive and possibly greater than the level of pollen-mediated gene flow between natural populations due to disparity in floral fecundity.

We used microsatellite markers and paternity exclusion analyses to directly measure and describe the levels of pollen immigration in remnant roadside populations of *A. saligna* subsp. *lindleyi* from a nearby planted stand of *A. saligna* subsp. *saligna*. We build on a previous study that assessed pollen-mediated gene flow patterns within a planted stand of subsp. *saligna* and pollen immigration into the planted stand from nearby trees of subsp. *lindleyi* (Millar et al. 2008a). In this study, we analysed the pattern of pollen-mediated gene flow into stands of subsp. *lindleyi* and compare results to that of the previous data set for subsp. *saligna*. Specifically we determined (i) the levels of pollen-mediated gene flow within and between remnant populations of subsp.

lindleyi, (ii) the relative levels of pollen immigration between a planted population of subsp. *saligna* and remnant populations of subsp. *lindleyi*, and (iii) the distances over which pollen-mediated gene flow occurs in subsp. *lindleyi*. We evaluate the results in light of the implications for management of risk associated with the extensive use of *A. saligna* for revegetation and agroforestry within its natural range.

Methods

Study species

Acacia saligna is a bushy shrub or tree, 2-10 m high at maturity that lives for up to twenty years under favourable conditions (Fox 1995). It is widespread in the southwest of Western Australia, where it occurs in a variety of habitats and is often found in degraded or disturbed environments (Doran and Turnbull 1997). It has a discontinuous distribution and often occurs in locally abundant patches at high stand density. *Acacia saligna* subsp. *lindleyi* is the most widespread of the four subspecies, occurring across most of the 'wheat and sheep' belt of southern Western Australia that is the target area for agroforestry. *Acacia saligna* subsp. *saligna* is restricted to the coastal areas of southern western Australia but produces the greatest biomass and has the best form for agroforestry purposes of the four subspecies. *Acacia saligna* subsp. *lindleyi* and subsp. *saligna* have mostly allopatric distributions with a very narrow zone of sympatry. Two other subspecies occur in the forest region to the south of the distribution of *A. saligna* subsp. *lindleyi*. *Acacia saligna* bears hermaphroditic flowers that are golden in colour and occur in many flowered, globular inflorescences. Trees flower in late winter/early spring and produce flat pods containing small, hard, black seeds (Maslin and McDonald 2004). Floral pollinators are thought to include a range of generalised insect species from the Hemiptera, Coleoptera and Diptera orders (Stone et al. 2003) and may include the European honeybee *Apis mellifera* (Sedgley et al. 1992).

Study site and sample collection

The study site was located south east of Toodyay, Western Australia (Fig. 4.1) within the natural distribution of *A. saligna* subsp. *lindleyi* where a planted stand of subsp. *saligna* occurred. The planted stand occupied approximately 0.55 hectares and contained 107 reproductively mature trees identified morphologically as subsp. *saligna*.

Remnant roadside trees of subsp. *lindleyi* occurred at varying distances from the planted stand. The closest remnant trees were directly across a road approximately 30 m from the edge of the planted stand. This remnant patch was quite dense with the number of individuals estimated at several hundred, although the majority of these were not reproductively mature at the time of sampling. The remnant patch extended away from the planted stand along a road verge in a westerly direction where trees became less abundant eventually occurring as individuals at a maximum distance approximately 330 m from the planted stand. Other remnant trees of subsp. *lindleyi* were present along the roadside extending away from the planted stand in a south easterly direction where they occurred sporadically in two main patches, roughly 900 m (approximately 30 trees) and 1500 m (approximately 50 trees) from the planted stand. Open pollinated seeds were collected from twenty nine selected maternal trees of *A. saligna* subsp. *lindleyi.* Trees of subsp. *lindleyi* had generally low flower and seed production, and trees with large seed crops were selected as maternal trees. These trees would have contributed the majority of the pollen pool and therefore represent the most likely pollen donors. The remaining trees had very low fecundity, would have made little contribution to the pollen pool and were not exhaustively genotyped. Seed was collected from 16 trees in the closest patch (TW01 – TW16), five trees from the patch at 900 m (TW17 – TW21) and eight trees from the most distant patch that commenced 1500 m from the planted stand (TW22 – TW29). All trees within the planted stand of subsp. *saligna* and the selected subsp. *lindleyi* trees were mapped using a differential global positioning satellite system.

DNA extraction and genotyping

DNA extraction from phyllodes and subsequent genotyping of trees within the planted stand of subsp. *saligna* trees and the selected subsp. *lindley*i trees has been described previously (Millar et al. 2008a). Seeds from maternal *lindley*i trees were germinated and genomic DNA extracted from seedling progeny. No seed germinated for two maternal *lindley*i trees and analysis was conducted for progeny of 27 maternal trees. Microsatellite markers for five loci were used to screen progeny arrays for the 27 families of subsp. *lindleyi* consisting of 1–25 seedlings (395 in total) as described previously (Millar et al. 2008a). Random sets of samples were rerun to check for repeatability of genotyping and segregation of maternal alleles in the progeny was checked.

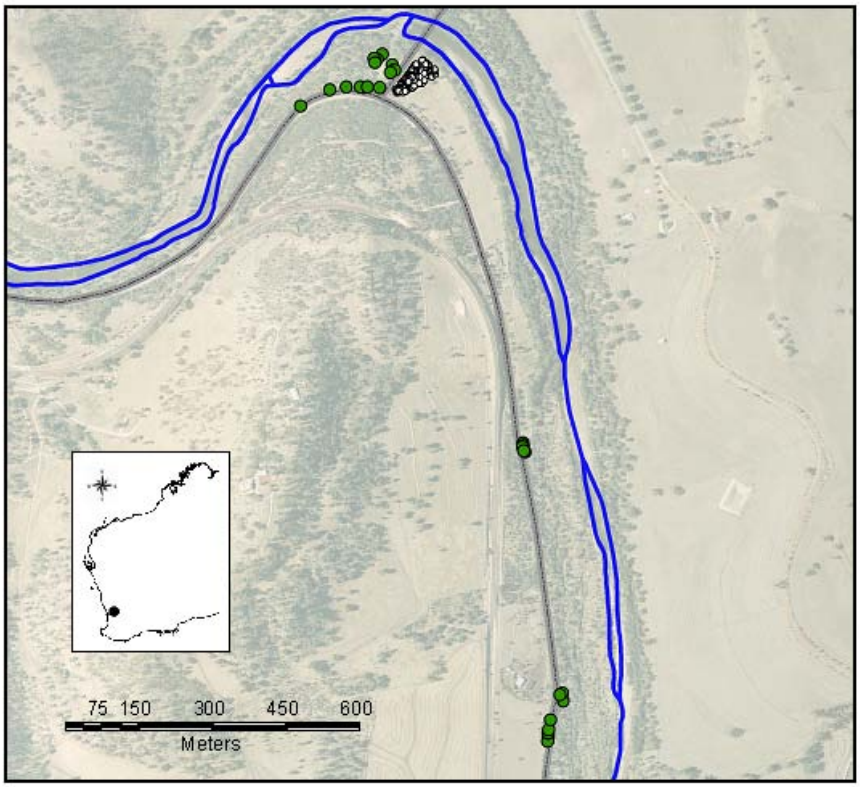

Figure 4.1 Map of the study site near Toodyay, Western Australia showing locations of sampled trees of *Acacia saligna*. Green circles represent sampled trees of subsp. *lindleyi*; open black circles represent sampled trees of subsp. *saligna* in the planted stand; broken grey lines represent roadways and the blue lines represent the Avon River.

Data analysis

Diversity parameters for the loci were determined separately for the 29 sampled trees of subsp. *lindleyi* and 107 sampled trees of subsp. *saligna* using CERVUS 2.0 (Marshall et al. 1998). Hardy Weinberg Equilibrium was tested for subsp. *lindleyi* trees using Fishers exact test as implemented in GENEPOP V3.4 (Raymond and Rousset 1995). Allele frequencies for the sampled populations of subsp. *lindleyi* and subsp. *saligna*, were calculated using GENALEX 6 (Peakall and Smouse 2006) and the overall co-ancestry coefficient (θ) for populations was calculated using FSTAT V2.9.3 (Goudet 2001).

Paternity assignment was conducted using maximum likelihood methods implemented in CERVUS 2.0 using 95% criterion for assignment and allowing mismatch at one locus. CERVUS 2.0 calculates a likelihood ratio for each candidate parent and compares the likelihood ratios of different candidate parents, using observed allele frequencies and taking into account possible typing errors. Simulation of parentage analysis was used to evaluate the confidence in parentage of the most likely candidate parent via a LOD score, which is the sum of the log-likelihood ratios at each locus. A negative LOD score implies that the candidate parent is less likely to be the true parent than an arbitrary randomly chosen individual, while a positive LOD score implies that the candidate parent is more likely to be the true parent than an arbitrary randomly chosen individual.

Since geitonogamous pollination is possible, assignment of paternity to the mother plant by CERVUS 2.0 was assumed to be due to selfing. The pattern of pollen-mediated gene flow within and between the remnant patches of *A. saligna* subsp. *lindleyi* was evaluated by determining the distance between mother and pollen source for each of the seed sired by trees of subsp. *lindleyi*. The number of actual pollen-mediated gene flow events was plotted as a function of distance between parental trees for 10 m distance classes. A number of curves known to describe pollen-mediated gene flow kernels, including normal, exponential, exponential power, Weibull, bivariate Student's *t* (2D*t*) and geometric (Austerlitz and Smouse 2002; Austerlitz et al. 2004), were fitted to the pollen-mediated gene flow data using SAS 9.1.3 statistical software (SAS Institute Inc 2006). The fit of each curve was compared using the pseudo R^2 statistic.

Results

Microsatellite marker utility

Parameters describing the utility of the five microsatellite markers for maternal trees of *A. saligna* subsp. *saligna* and subsp. *lindleyi* are presented in Table 4.1. The microsatellite loci were moderately variable with up to 13 alleles per locus. The loci showed similar levels of polymorphism in both subspecies (Table 4.1), although the number of alleles (*A*) in the planted stand of subsp. *saligna* was greater as might be expected if seed was sourced from several populations.

Table 4.1 Details of polymorphism and utility of five microsatellite loci for trees of *Acacia saligna* subsp. *lindleyi* and trees of *Acacia saligna* subsp. *saligna*.

	subsp. *lindleyi*						subsp. *saligna*					
Locus	*A*	H_o	H_e	PIC	Excl (2)	Null	*A*	H_o	H_e	PIC	Excl (2)	Null
As029	2.0	0.414	0.334	0.274	0.137	-0.114	5.0	0.421	0.427	0.392	0.232	-0.0153
Am012	5.0	0.964	0.658	0.579	0.372	-0.212	10.0	0.785	0.792	0.763	0.603	0.0000
Am030	9.0	1.000	0.864	0.831	0.699	-0.085	9.0	0.425	0.470	0.442	0.279	0.0726
As137	5.0	0.759	0.641	0.587	0.398	-0.120	5.0	0.364	0.356	0.337	0.199	-0.0145
Am367	6.0	0.724	0.546	0.489	0.309	-0.191	13.0	0.738	0.882	0.865	0.754	0.0839
Mean	5.4	0.772	0.609	0.552	0.932*	-0.144	8.4	0.547	0.585	0.560	0.956*	0.0253

A: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: polymorphic information content; Excl (2): power of exclusion for the second parent; Null: estimated null allele frequency.

*The total probability of exclusion for the second parent over all five loci is given.

All parameters were calculated using CERVUS 2.0 (Marshall et al. 1998).

Heterozygosity was high in both subspecies. Significant departure from Hardy-Weinberg equilibrium for all loci was not detected. Null allele frequency averaged – 0.144 for trees of subsp. *lindleyi* and 0.0253 for trees of subsp. *saligna*. As mismatches generated by null alleles are treated as typing errors in paternity assignment, null alleles with low frequencies do not have a significant effect. The polymorphic information content (PIC) of the loci was high and the total exclusion probability over the five loci was 0.932 for the second parent for analysis of subsp. *lindleyi* and 0.956 for analysis of subsp. *saligna*.

Population differentiation

A high level of genetic divergence was found for the sampled populations of subsp. *saligna* and subsp. *lindleyi*. Allele frequencies for five microsatellite loci are shown in Table 4.2. Four private alleles not present in the sampled trees of subsp. *saligna* were detected in subsp. *lindleyi* trees. One of these alleles (at locus Am367) was of moderate frequency for subsp. *lindleyi* and the remaining three alleles were of low frequency. Trees in the stand of subsp. *saligna* had 19 private alleles not detected in sampled trees of subsp. *lindleyi*. Three of these, one at locus As029 and two at locus Am012, were high frequency alleles in subsp. *saligna*. The remaining 16 private alleles were of low frequency in subsp. *saligna*. An overall co-ancestry coefficient (θ) of 0.239 also indicates a high level of genetic divergence between the two populations.

Paternity assignment

Paternity assignment identified a pollen source for the majority of seed from all maternal trees. Using the five loci, a most likely father (positive LOD score) was assigned for 96.5% of progeny from seed crops of subsp. *lindleyi* trees. A single most likely male parent was not assigned to fourteen progeny. These progeny were assigned equal LOD scores for two or more sampled trees and as a result were excluded from further parentage and distance analysis. A single most likely pollen source from sampled trees of subsp. *lindleyi* was determined for 255 progeny (64.5%) overall, and a single tree in the planted stand of subsp. *saligna* was identified as a most likely pollen source for 126 progeny (32%, Table 4.3). Fifteen selfing events were detected among the progeny of subsp. *lindleyi* trees.

Table 4.2 Allele frequencies for sampled trees of *Acacia saligna* subsp. *lindleyi* and sampled trees of *Acacia saligna* subsp. *saligna* for five microsatellite loci. Private alleles are indicated in bold.

Locus	Allele	Frequency	
		subsp. *saligna*	subsp. *lindleyi*
As029	134	0.136	0.793
	143	0.009	0.000
	146	0.738	0.207
	150	0.009	0.000
	155	0.107	0.000
Am012	100	0.089	0.357
	104	0.028	0.446
	106	0.005	0.000
	108	0.131	0.000
	110	0.168	0.018
	112	0.364	0.161
	114	0.150	0.000
	116	0.014	0.000
	118	0.005	0.018
	122	0.047	0.000
Am030	71	0.009	0.138
	79	0.005	0.017
	82	0.009	0.207
	84	0.000	0.052
	86	0.009	0.224
	88	0.009	0.000
	90	0.123	0.069
	92	0.712	0.138
	94	0.094	0.069
	96	0.028	0.086
As137	123	0.089	0.121
	125	0.061	0.086
	127	0.000	0.034
	133	0.005	0.207
	137	0.794	0.552
	143	0.051	0.000
Am367	68	0.000	0.224
	69	0.093	0.000
	70	0.173	0.069
	71	0.037	0.000
	72	0.201	0.638
	73	0.047	0.000
	75	0.000	0.017
	76	0.117	0.017
	79	0.070	0.000
	80	0.042	0.000
	81	0.089	0.000
	82	0.107	0.034
	83	0.005	0.000
	84	0.014	0.000
	85	0.005	0.000

Table 4.3 Pollen sources in remnant patches of *Acacia saligna* subsp. *lindleyi*. Gene flow events and distances are average values for maternal trees in each patch of *A. saligna* subsp. *lindleyi*, and for all maternal trees of subsp. *lindleyi* from all patches.

Subsp. *lindleyi* patch	Distance from subsp. *saligna* (m)	Pollen source (% of progeny)				Unassigned (%)	Inter-subspecific gene flow distance (m)	Within subspecies gene flow distance (m)
		TW01-TW16	TW17-TW21	TW22-TW29	subsp. *saligna*			
TW01-TW16	30-330	49.5	8	8	33	1.5	112 (52)	381 (316)
TW17-TW21	867-955	28	39	14	5	14	922 (-)	519 (160)
TW22-TW29	1462-1650	21.5	20	11.5	40	7	1580 (25)	997 (234)
All trees	~632	42.5	13	9	32	3.5	451 (384)	454 (568)

Values in parenthesis are standard errors

Pollen-mediated gene flow

Inter and intra-subspecific pollen immigration into remnant patches of *A. saligna* subsp. *lindleyi* was extensive, accounting for 55% of outcrossed progeny and occurring over large distances of up to 1607 m. This pollen immigration included high levels of inter-subspecific hybridisation from the planted stand of subsp. *saligna*. Overall, 32% of all assayed progeny from trees of subsp. *lindleyi* resulted from hybridisation with pollen from trees of subsp. *saligna* (Table 4.3). Inter-subspecific pollen immigration distances ranged from 42 m to 1607 m. The average distance of inter-subspecific pollination events in subsp. *lindleyi* (451 m) was similar to the average distance of pollination events for progeny sired only by other trees of subsp. *lindleyi* (454 m) (Table 4.3). Nearly half (42%) of the subsp. *saligna* trees in the planted stand contributed pollen to progeny of subsp. *lindleyi* trees. Inter-subspecific pollen immigration was highly variable among the sampled trees of subsp. *lindleyi*, ranged from 0 to 100% for individual trees and was not related to distance from the planted stand.

The pattern of pollen immigration was variable for the three patches of subsp. *lindleyi* (Table 4.3). Trees in the largest patch (TW01 – TW16) received the majority of outcrossed pollen from within the patch (49.5%) and from the nearby planted stand of subsp. *saligna* (33%), and relatively low intra-subspecific pollen immigration from the other two patches of subsp. *lindleyi* (8% and 8%). The middle patch of remnant *A. lindleyi* tress (TW17-TW21) received the majority of outcrossed pollen from within the patch (39%). Levels of intra-subspecific pollen immigration from the other two patches of subsp. *lindleyi* increased (28% and 14%). This patch received the lowest amount of inter-subspecific pollen immigration from the planted stand of *A. saligna* (5%). The most isolated patch of subsp. *lindleyi* had the lowest level of within patch mating (11.5%), high levels of pollen immigration from the other two patches of subsp. *lindleyi* (21.5% and 20%) and a high level of inter-subspecific pollen immigration from the planted stand of subsp. *saligna* (40%).

The frequency distribution of all outcrossing events over dispersal distance for progeny of subsp. *lindleyi* is depicted in Figure 4.2. A geometric curve provided the best fit to the pollen-mediated gene flow data. Using a pseudo R^2, the geometric distribution explained over one third (36%) of the trend seen in pollen-mediated gene flow and describes a fat tailed pollen dispersal curve.

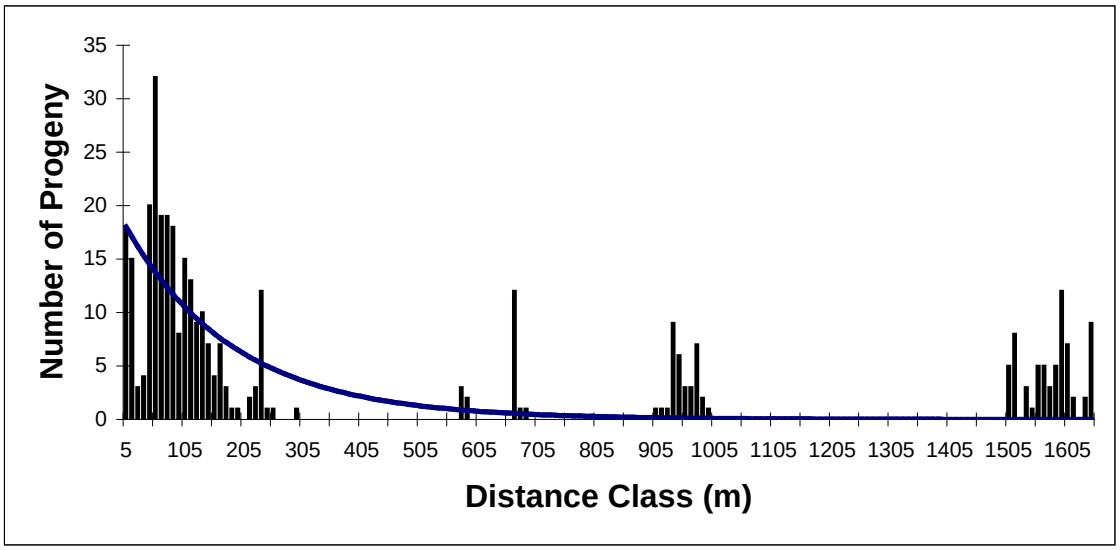

Figure 4.2 Distribution of outcrossed pollination events for maternal trees of *Acacia saligna* subsp. *lindleyi*. The fitted geometric pollen-mediated gene flow curve is shown.

Discussion

This investigation of pollen-mediated gene flow in *A. saligna* has shown a high level of intra-subspecific gene flow between remnant patches of subsp. *lindleyi*, some of which occurred over distances greater than 1500 m. A high level of inter-subspecific pollen immigration into remnant patches of subsp. *lindleyi* from a planted stand of subspecies *saligna* was also detected, with pollen immigration again over distances greater than 1500 m. A fat tailed pollen dispersal curve described the extensive long distance pollen-mediated gene flow. The results indicate that gene flow may be acting to maintain genetic continuity among remnant populations of subsp. *lindleyi*. They also indicate that remnant populations may be placed at risk from considerable levels of inter-subspecific gene flow via pollen immigration from introduced populations, even when separated by distances of 1500 m.

Pollen-mediated gene flow among remnant patches

This study showed a high frequency of intra-subspecific gene flow at extensive distances, in fragmented remnant roadside populations of a temperate woody perennial species. Patterns of extensive long distance pollen-mediated gene flow have been reported previously for a number of tropical and neo tropical tree species occupying highly fragmented landscapes as well as one temperate Australian tree species (Stacy et

al. 1996; Nason and Hamrick 1997; Kaufman et al. 1998; White et al. 2002; Dick et al. 2003; Byrne et al. 2007). The results of these studies indicate an extensive network of gene exchange between fragmented remnants and isolated trees. This is contrary to classical population genetic theory that may predict reduced gene flow for physically isolated populations (Slatkin 1987; Young et al. 1996). High levels of gene flow occurring over large distances, coupled with the highly outcrossing mating systems generally found for tree species, are likely to maintain large effective population sizes, high population genetic diversity, and continuity between fragmented remnant populations. The extensive pollen-mediated gene flow observed here in *A. saligna* may be sufficient to mitigate the presumed effects of reduced population size and isolation, that can result in depleted population genetic variation and a significant decrease in reproductive performance (Ellstrand and Ellam 1993). Despite the fragmented distribution of *A. saligna* subsp. *lindleyi*, inter-patch gene flow is extensive and it is unlikely that significant levels of inbreeding are occurring in populations in the wheatbelt of southern Western Australia. The extensive pollen-mediated gene flow found in *A. saligna* supports the view that decreased pollen-mediated gene flow should not always be expected in fragmented landscapes, especially for entomophilous tree species (Byrne et al. 2007).

Inter-subspecific hybridisation

While the majority of inter-subspecific pollen immigration from introduced plantings of subsp. *saligna* is likely to occur over short distances, substantial amounts of inter-subspecific hybridisation may occur in subsp. *lindleyi* even when introduced populations are located at least 1500 m away. The pollen-mediated gene flow distances observed are truncated, due to the limited occurrence of *A. saligna* subsp. *lindleyi* within the study area and inter-subspecific pollen-mediated gene flow may occur over distances greater than 1500 m. The average distance over which immigrant pollen travels may be similar to average intra-subspecific pollen-mediated gene flow distances at a given site.

Pollen contamination between introduced and natural remnant populations requires both pollen-mediated gene flow, and effective fertilisation to produce viable seed. The high level of inter-subspecific hybridisation detected between subspecies of *A. saligna* indicates that the subspecies are highly inter-fertile and produce viable hybrid seed

capable of germination. However, a disparity in inter-subspecific hybridisation between the two subspecies of *A. saligna* was observed. The level of pollen immigration into remnant patches of subsp. *lindleyi* was considerably greater than the level of pollen immigration from subsp. *lindleyi* remnants into the planted stand of subsp. *saligna* detected previously by Millar et al. (2008a). Paternity assignment for progeny of nine maternal trees in the planted stand of subsp. *saligna* identified trees in the remnant patches of subsp. *lindleyi* as the pollen source for 14% of progeny (Millar et al. 2008a). This was not due to lack of exhaustive genotyping of trees of subsp. *lindleyi* as the pollen source for the remainder of the progeny of subsp. *saligna* was identified from trees within the planted stand.

The disparity in inter-subspecific pollen immigration is likely due to differences in the total amount of pollen produced by each subspecies. Pollen production for individual trees is influenced by size, form, and floral density, and at population level is influenced by population size (Kwak et al. 1998). Populations with poor flowering and pollen production are likely to experience a relatively large proportion of pollen immigration compared to populations that are more fecund. *Acacia saligna* subsp. *lindleyi* has low floral fecundity as it has a spindly form, produces relatively few terminally located flowers and has low seed set. In contrast, trees of subsp. *saligna* show the greatest biomass, floral density and seed set of all the subspecies of *A. saligna* (George 2005). Although relative floral fecundities have not been formally assessed the disparity in floral fecundity between subsp. *lindleyi* and subsp. *saligna* was noticeable at the study site where it would have resulted in trees of subsp. *saligna* producing substantially more pollen in comparison to trees of subsp. *lindleyi* and lead to a greater contaminant pollen source for subsp. *lindleyi* as compared to subsp. *saligna*. Temporal variation in flowering intensities and floral fecundity may also occur at the site resulting in annual variation in relative amounts of pollen. The results presented here are based on a single seed cohort and therefore represent gene flow for a single season only.

Flowering intensities, floral fecundity and pollen production may also be affected by the age composition of populations that will vary at different sites. All trees in the planted stand at the study site were mature and flowered profusely during the year of seed collection while the remnant populations were comprised of trees of various ages, including many juveniles that produced relatively few flowers and presumably little

pollen. While the two subspecies rarely occur in sympatry under natural conditions, there is a high likelihood that large populations of subsp. *saligna* will be planted throughout the natural range of subsp. *lindleyi* for revegetation purposes. These results show that high levels of pollen-mediated gene flow can be expected from plantings of subsp. *saligna* once trees reach maturity.

At present, the impacts of inter-subspecific gene flow and the fitness of hybrid progeny relative to parental genotypes have not been assessed for *A. saligna*. The impacts of outbreeding depression are highly unpredictable for a given taxon but adverse effects may occur even when levels of inter-subspecific gene flow are low (Edmands and Timmerman 2003). Assessment of outbreeding depression in hybrid progeny in *A. saligna* will require fitness measurements over the entire life cycle of hybrid progeny and extension of hybridisation studies beyond the first generation to include backcross as well as F_2 hybrids. The likelihood, and the long-term genetic and ecological consequences, of introgression in *A. saligna* are also unknown and require investigation.

Pollinator behaviour

Pollen-mediated gene flow in entomophilous species is directly related to pollinator behaviour. Bees and small generalist insect pollinators are capable of effecting long distance pollen-mediated gene flow (Levin and Kerster 1969; White et al. 2002). Pollinator behaviour is related to floral density (Levin and Kerster 1969; Kwak et al. 1998) with flight distances of insect pollinators expected to increase as population density and overall floral production decreases (Kwak et al. 1998). The proportion of within patch fertilisation events decreased as patches of subsp. *lindleyi* became less dense and more spatially isolated. Foraging insect pollinators may spend less time within remnant patches as overall floral density decreases, effecting fewer intrapopulation pollinations than in larger, denser or more florally fecund populations (Murawski and Hamrick 1991; Stacy et al. 1996). Low floral density in *A. saligna* subsp. *lindleyi* also appears to promote long interpopulation pollinator flights leading to high levels of pollen-mediated gene flow among remnant populations of *A. saligna*.

Recommendations for management practices

We have demonstrated an extensive level of long-distance inter-sub-specific gene flow between two highly genetically divergent subspecies of the *A. saligna* species complex,

subsp. *lindleyi* and subsp. *saligna*. This is an important finding with regards to ensuring the development of agroforestry within Western Australia is environmentally sustainable, for the management of natural resources and the conservation of natural populations. It is likely that further anthropogenic disturbance via the large-scale planting of divergent populations of subsp. *saligna* will generate considerable amounts of contaminant pollen in the vicinity of fragmented natural remnants of subsp. *lindleyi* in the Western Australia wheatbelt. The risk of extensive pollen immigration into remnant populations can be reduced through cultural management practices, such as restricting germplasm used in agroforestry populations to that with flowering times that do not overlap with those of natural populations or harvesting trees planted for agroforestry before flowering and pollen production commences. In *A. saligna*, the flowering times of all subspecies overlap so the first option may be difficult to implement in the short term. Selection for early flowering genotypes in subsp. *saligna*, which has the earliest flowering of the subspecies, may result in reduced overlap in flowering times among the subspecies. The second option of harvesting before floral maturity may be problematic for long lived species, however, *A. saligna* is being promoted as a short rotation coppice crop (Bartle et al. 2002) that may be harvested before trees commence flowering and pollen production.

The most reliable short-term practice to limit pollen-mediated gene flow in *A. saligna* may include isolation of agroforestry crops from small natural populations by distance or by the use of barrier or guard rows. Guard rows are comprised of an alternative species and used to surround crops to reduce the physical movement of pollen and 'trap' pollinators thus reducing their movement directly from the crop. The effectiveness of guard rows has not been investigated at this stage therefore the most effective option may be isolation, and isolation distances greater than 1.5 km are recommended to prevent significant levels of gene flow into natural remnant populations.

Acknowledgements

We thank Bronwyn Macdonald, Maggie Hankinson and Lin Wong for assistance in the laboratory and Wayne O'Sullivan for assistance with morphological identification at the study site.

Chapter Five
Characterisation of polymorphic microsatellite DNA markers for *Acacia saligna* (Labill.) H.L.Wendl. (Mimosaceae)

Statement of Contribution

M. A. MILLAR

Conducted all laboratory work, all data and results analysis, wrote the final paper and acted as corresponding author

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M. BYRNE

Provided assistance with laboratory techniques and writing the final paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

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Chapter Five

Characterisation of polymorphic microsatellite DNA markers for *Acacia saligna* (Labill.) H.L.Wendl. (Mimosaceae)

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Keywords: enriched library, microsatellites, diagnostic markers, agroforestry, *Acacia saligna*

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Running Title: *Microsatellite DNA markers for* Acacia saligna

Abstract

A genomic library was constructed and ten novel polymorphic microsatellite markers developed for *Acacia saligna*, a species complex being developed for agroforestry across southern Australia. Polymorphism was investigated for these markers in 30 individuals from two subspecies. Alleles per locus averaged 4.0 for subsp. *saligna* and 5.2 for subsp. *lindleyi*. Population specific alleles were detected for all loci. The microsatellite markers will be used for diagnostic assessment of the four main subspecies of the species complex.

Acacia saligna (Labill.) H.L.Wendl. (Mimosaceae) is a bushy shrub or tree native to southern Western Australia where it occurs in local abundance across a widespread range (Maslin and McDonald 2004). The species has been widely planted both in Australia and overseas for a range of purposes, including for firewood and fodder, and for windbreak and amenity planting. The species complex is described by four main intraspecific variants to be erected as sub-species (McDonald and Maslin in prep). *Acacia saligna* is being developed for agroforestry across the low rainfall areas of southern Australia where large-scale revegetation is required to combat dryland salinity. Microsatellite markers were developed for *A. saligna* as part of a larger effort in breeding and conservation programs.

Microsatellite primers were developed from a specimen of *Acacia saligna* subsp. *saligna* (ms). Genomic DNA was extracted from adult phyllodes using a modified cetyltrimethyl ammonium bromide (CTAB) extraction method (Byrne et al. 1993) with the addition of 0.1 M sodium sulfite to the extraction and wash buffers (Byrne et al. 2001). A hybridisation capture approach was used to construct a highly enriched genomic DNA library (Glenn and Schable 2005). Genomic DNA (20µg) was digested with *RsaI* and ligated onto SuperSNX linkers (New England BioLabs, Inc). Linker ligated DNA was hybridised with biotinylated dinucleotide microsatellite probes (AC_{13} and AG_{13}) and enriched with a Streptavidin M-280 Dynabead (Dynal, Oslo, Norway) enrichment protocol. Enriched DNA was recovered by Polymerase Chain Reaction (PCR) amplification, ligated into vector pCR®4-TOPO® and transformed into competent *Escherichia coli* TOP10 cells using a TOPO TA Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer's protocol. Resulting recombinants were selected using ampicillin and amplified by PCR. Amplified products were hybridised onto Hybond-N+ membranes using a Bio-Dot® microfiltration apparatus (Bio-Rad) and labelled with biotin labelled dinucleotides. Detection of labelled probes was performed with the chemiluminescent CDP-*Star*™ Universal Detection (Tropix) kit detection method. After washing and blocking, membranes were incubated with Streptavidin-Alkaline Phosphatase conjugate, washed and incubated in CDP-Star Substrate then exposed to radiography film.

DNA inserts from hybridisation positive colonies were purified using ExoSAP (Glenn and Schable 2005) and sequenced using BigDye Terminator chemistry. Of the 64

clones sequenced, 42 contained a microsatellite sequence of eight or more repeats. Sequences flanking the microsatellite repeat motifs were analysed and compatible primer sequences were identified with PRIMER3 (Rozen and Skaletsky 2000). Primer pairs were tested for mispriming and primer dimers with AMPLIFY 1.2 (Engels 1993). Primer pairs were developed for 20 microsatellite repeats.

Microsatellite loci were amplified in a total volume of 15 μl per reaction containing 20 ng template DNA, 50 mM KCl, 20mM Tris HCl pH 8.4, 0.2 mM each dNTP, 0.3 μM of each primer, 0.5 units of *Taq* DNA polymerase and variable $MgCl_2$ concentration depending on the primer as detailed in Table 5.1. PCR amplification conditions were 96°C for 2 min, 30 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C followed by 5 min at 72°C. Reactions were completed in a Touchdown Thermal Cycler (Hybaid Ltd).

Table 5.1 Primer sequence, GenBank Accession No., Repeat units and magnesium chloride concentration for amplification of 10 microsatellite loci in *Acacia saligna*.

Locus	Primer sequence (5′-3′)	GenBank Accession No.	Repeat	$MgCl_2$ (mM)
As2.04	F: CAAATGAAAAAGAATGCTTGGTG R: CATCTTGTTAAGGAATATTGGTTTCG	EF194133	$(TC)_9$	1.0
As2.13	F: CGTACCAAATTGCTC CTTAAC C R: TCCTGCCAAACATGAAAG C	EF194134	$(AG)_8(TA)_5$	1.5
As2.17	F: TCCTCGCTTCTCGACATTTT R: GCTCGAACCTTTCAAACGAA	EF194135	$(AC)_7(TC)_7$	1.0
As2.20	F: TTAGTGAAATGCGACAGAGAGAC R: CATAGCCTGGCAAATCCTG	EF194136	$(AG)_7$	1.0
As2.34	F: ACGGCCCTCGTTAGTCTG R: CTTGAACACCCCATGTGC	EF194137	$(TC)_9$	1.5
As2.46	F: GTTCTCTTGCCCTGTTTGCT R: AGGCTGGAAATAAATGGAGGA	EF194138	$(TC)_7$	1.5
As2.47	F: CTCAGGTCCAAGAGGAACAAG R: TTAGTGAATAGGTGGGAAATGG	EF194139	$(TC)_8$	1.5
As2.57	F: GGAAGAAGAGAATAGAGAAGAAAAAGA R: CACCCTACCCCTGCCAAT	EF194140	$(AG)_{12}$	1.0
As2.61	F: CTGAATGTGCTTCTTCTCTCTTGG R: GGGAATCTGCCTTTAGTTTGC	EF194141	$(TC)_{12}$	1.5
As2.62	F: GGATTTGCCATTTATTACCTACAAG R: GCTACACTCCCTCTTCCATTG	EF194142	$(TC)_6(CA)_{10}$	1.5

Loci were initially screened for polymorphism in eight individuals on 8% non-denaturing polyacrylamide gels and stained with ethidium bromide. Of the 20 primers trialled, ten produced amplified products that could be interpreted as single loci. The

remaining primers appeared to amplify multiple loci that could not be resolved even with high stringency conditions. Genetic diversity was investigated further in two populations, one of subsp. *saligna* (ms) and one of subsp. *lindleyi* (ms). Fifteen individuals were genotyped from each population. PCR conditions were as above except the size of PCR products was determined by automated fluorescent scanning detection using an Applied Biosystems 3730 DNA Analyser (Applied Biosystems) and Genemapper™ v3.7 analysis software (Applied Biosystems).

Tests for linkage disequilibrium (LD) were conducted using GENEPOP version 3.4 (Raymond and Rousset 1995). Tests for Hardy-Weinberg equilibrium (HWE) were conducted and null alleles and genetic diversity parameters assessed using CERVUS 2.0 (Marshall et al. 1998) (Table 5.2). No significant linkage association was found among the loci. A significant deviation from HWE was detected at loci As2.13 and As2.34 ($p<0.05$). Disequilibrium may be due to null alleles detected at frequencies of 0.578 and 0.576 at these loci. Null alleles were detected at low frequency (ranging from 0.109 to 0.436) for all other loci. The number of alleles per locus averaged 4.0 for subsp. *saligna* and 5.2 for subsp. *lindleyi*. Population specific alleles were found at each locus. For example, although locus As2.13 was monomorphic for allele 125 in subsp. *saligna* this allele was not present in the other subspecies. Selected microsatellite markers are currently being used for diagnostic assessment of the four subspecies of *A. saligna*.

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Table 5.2 Diversity characteristics of ten microsatellite loci for 30 individuals (15 each) of *Acacia saligna* subsp. *saligna* (ms) and subsp. *lindleyi* (ms).

	Population							
	subsp. *saligna* (ms)				subsp. *lindleyi* (ms)			
Locus	N_a	Allele size	H_o	H_e	N_a	Allele size	H_o	H_e
As2.04	5.0	172-194	0.533	0.662	6.0	172-212	0.333	0.779
As2.13	1.0	125	0.000	0.000	2.0	129 – 133	0.333	0.370
As2.17	4.0	119 - 134	0.133	0.680	11.0	130 – 199	0.933	0.878
As2.20	4.0	122 - 129	0.667	0.678	3.0	119 – 126	0.143	0.265
As2.34	6.0	251-264	0.308	0.520	2.0	251-257	0.067	0.067
As2.46	1.0	106	0.000	0.000	3.0	106-108	0.400	0.660
As2.47	6.0	146 - 161	0.733	0.685	4.0	144 – 159	0.533	0.536
As2.57	5.0	142-153	0.600	0.736	6.0	144-153	0.000	0.833
As2.61	3.0	235 - 243	0.200	0.191	10.0	218 – 239	0.800	0.878
As2.62	5.0	164-173	0.667	0.713	5.0	163-176	0.429	0.381
Mean	4.0		0.384	0.486	5.2		0.3971	0.565

N_a, number of alleles; Allele size, range of allelic fragment sizes in base pairs; H_o, observed heterozygosity; H_e, expected heterozygosity.

Chapter Six
A rapid PCR based diagnostic test for the identification of subspecies of *Acacia saligna*

Statement of Contribution

M. A. MILLAR

Conducted all laboratory work, all data and results analysis, wrote the final paper and acted as corresponding author

Signed Date

M. BYRNE

Provided assistance with laboratory techniques and writing the final paper.

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

Signed Date

I. NUBERG

Reviewed and provided comment on final drafts of the paper

I give consent for M.A. Millar to present this paper for examination towards the Doctor of Philosophy

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M. SEDGLEY

Reviewed and provided comment on final drafts of the paper.

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Signed Date

Chapter Six

A rapid PCR based diagnostic test for the identification of subspecies of *Acacia saligna*

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Key words: microsatellite, diagnostic tool, Acacia saligna, *species complex, genetic differentiation*

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Abstract

Subspecific taxa of species complexes can display cryptic morphological variation and individuals and populations can often be difficult to identify with certainty. However, accurate population identification is required for comprehensive conservation and breeding strategies, and for studies of invasiveness and gene flow. Using five informative microsatellite markers and a Bayesian statistical approach, we developed an efficient PCR based diagnostic tool for the rapid identification of individuals and populations of the *Acacia saligna* species complex of Western Australia. We genotyped 189 individuals from 14 reference populations previously characterised based on morphology and used this data to investigate population structure in the species complex. High total genetic diversity (H_T = 0.729) and high population differentiation (θ = 0.355) indicated strong interspecific structuring. With the provision of prior population information, the reference data set was optimally resolved into four clusters, each corresponding to one of the four main proposed subspecies, with very high membership values (Q > 97%). The reference data set was then used to assign individuals and test populations to one of the four subspecies. Assignment was unequivocal for all test individuals from two populations of subsp. *lindleyi* and for all but one individual of subsp. *stolonifera*. Individuals from populations of subsp. *saligna* and subsp. *pruinescens* showed a degree of genetic affinity for the two subspecies in their assignments although the majority of individuals were correctly assigned to subspecies. The diagnostic tool will assist in characterising populations of *A. saligna*, especially naturalised and invasive populations of unknown origin.

Introduction

Species are considered the fundamental taxonomic units of biology, and accurate and efficient identification of species is generally required prior to further scientific investigations in ecology, conservation or breeding. Identification and taxonomic classification of species has traditionally been based on their morphological description, although the development of barcoding projects may lead to identification of species based on DNA profiles (Herbert et al. 2004). In situations of morphological complexity, phylogenetics is being applied to assist in delineation of species boundaries in both plants (Broadhurst et al. 2004; Elliott and Byrne 2004) and animals (Eldridge and Close 1992; Crowther et al. 2002). These studies take a population genetic approach that uses structuring of genetic diversity to identify cohesive biological groups indicative of species. The effectiveness of this approach has recently improved because of the combination of more readily available informative molecular markers, such as microsatellites, and the power of statistical inference, such as maximum likelihood assignment procedures (Paetkau et al. 1995; Rannala and Mountain 1997) and Bayesian clustering models (Pritchard et al. 2000) that use genotyping data to provide an *a posteriori* delimitation of existing genetic structure.

In addition to addressing fundamental questions of biological entities and relationships, a population genetics approach can be used to create diagnostic tools. Indeed, molecular genetics is readily used for identification of fungal samples where morphological identification can be tedious or expensive (McCartney et al. 2003; Atkins and Clark 2004). Recently, Duminil et al. (2006) suggested that a similar approach could be taken for identification of blind samples of tropical rainforest trees for population genetic surveys. Routine morphological identification is difficult in these tall tree species due to the diagnostic floral characters only being available at certain times of the year (i.e. when the tree is flowering) and being held high in the canopy. Thus, population genetic methodology can be used to develop diagnostic tools for the identification of biological entities where morphological identification is difficult or unreliable for whatever reason. This approach may be particularly valuable where individuals occur outside native populations, such as in plantings or in invasive populations.

In this study, we demonstrate the utility of a population genetics based approach to creating a diagnostic tool for taxon identification with the example of a woody perennial species complex, *Acacia saligna*. *Acacia saligna* is a native Australian species complex, composed of up to five proposed subspecies that will be formally described in a taxonomic revision (Maslin et al. 2006). The species complex shows great variation in morphological, ecological and biological attributes over its natural distribution. The subspecies can be distinguished by a combination of morphological differences including phyllode appearance, the shape of the inflorescence bud, the length of racemes and the diameter, colour and number of flower heads (M McDonald, *pers. comm*.); however, these characteristics can only be assessed when plants are suitably mature and then only while plants are developing buds or flowering. Population identification is also exacerbated by morphological variation due to the biological age of the trees, which exhibit variable juvenile growth and changes in phyllode colour displayed over seasons.

The species has been planted extensively for revegetation programs throughout its natural range, making the delineation of subspecies ranges problematic and hampering the identification of both natural and introduced populations. In addition, the species is an invasive weed in areas outside its native range in south-eastern Australia and in other countries such as South Africa (Henderson 2001), Chile (Midgley, Christoludou cited in Maslin and McDonald 2004), Cyprus (Georgiades 1994; Hadjikyriakou and Hadjisterkotis 2002; Christodloulou 2003), Spain (Dana et al. 2001; Sanz-Elorza et al. 2001) and Portugal (De la Lama cited in Fox 1995). The subspecies of *A. saligna* display variation in key traits, such as seed set, fecundity and suckering that are important aspects of conservation, breeding and weed control programs, therefore it is frequently important to know the subspecies identity of the individuals or populations being investigated (Byrne and Broadhurst 2002; Byrne and Millar 2004). Previous genetic analysis conducted on RFLP data using phylogenetic techniques revealed high levels of genetic differentiation between populations of each of the four subspecies (θ = 0.359) indicating strong genetic structure within the species (George et al. 2006). Thus, it appears feasible to develop a diagnostic test for subspecies identification based on genetic structure.

In this study, we developed a rapid microsatellite based diagnostic tool for more accurate identification of individuals of each of the four main subspecies of *A. saligna.* Accurate identification of taxa is vital for the development of comprehensive *in situ* conservation strategies, for germplasm collections aimed at *ex situ* conservation and the establishment of breeding and domestication programs, for the identification of naturalised and invasive populations, and for studies of gene flow, hybridisation and introgression between the subspecies. Specifically, we constructed a 'reference' data set using data obtained by genotyping a number of populations that had undergone prior morphologically assessment (George et al. 2006) with five microsatellite markers previously developed for use in *A. saligna* (Millar and Byrne 2007). Bayesian assignment analysis of multilocus nuclear genotypes was used to identify the optimal number of genotypic clusters for the reference data set. Structuring within the reference set was then used to assign individuals from a number of 'test' populations to their corresponding genotypic clusters. Test populations were selected from those where subspecies identification was uncertain and accurate identification desired.

Materials and Methods

Study species

Acacia saligna is a diploid insect pollinated species complex with a long history of utilisation, mainly for forage and fuel wood, both in Australia and overseas (Midgley and Turnbull 2003). The species has recently been identified as a high priority for development for agroforestry across the southern agricultural areas of Australia (Maslin and McDonald 2004; Olsen et al. 2004) where planting of deep rooted perennial species is required to ameliorate the impacts of land degradation through dryland salinity. The work presented here focuses on four of the five proposed subspecies of *A. saligna*, subsp. *saligna*, *stolonifera*, *pruinescens* and *lindleyi* (George 2006; Maslin et al. 2006). A fifth subspecies, subsp. *osullivaniana*, has recently been discovered however it occurs in a very limited distribution north of Perth and there is uncertainty regarding its relationship to the other taxonomic entities.

Acacia saligna subsp. *saligna* is restricted to the Swan Coastal Plain around Perth, has a robust main stem, smooth, reddish bark, produces suckers and coppices well and is a dense and bushy tree with high biomass production. It has relatively narrow dull phyllodes, obtuse inflorescence buds and long racemes. *Acacia saligna* subsp.

stolonifera occurs mainly in the forest region and west to the coast, usually has a single stem, friable bark, suckers strongly, and produces dull phyllodes, conical inflorescence buds and short racemes. *Acacia saligna* subsp. *pruinescens* has a limited distribution around Bridgetown-Kojonup-Manjimup and its distribution overlaps with subsp. *stolonifera*. It typically has large, crooked, white pruinose stems, friable bark and suckers strongly, and produces glaucous, dull, pruinose phyllodes, and conical inflorescence buds. *Acacia saligna* subsp. *lindleyi* is the most widespread of the subspecies occurring mainly in the inland wheatbelt areas and on southern sand dunes. It has straight stems, smooth bark, little or no propensity for suckering and trees can be spindly with little biomass. Trees have green shiny phyllodes, conical inflorescence buds and limited terminal flowering.

Sample populations

Populations at well-characterised sites representative of the morphological variation present in the species were used as reference populations (Table 6.1). Each population was comprised of one subspecies only with no suspected admixture or hybrid progeny present. The populations at Settlers Hill, Flynn, Yunderup, Meelup, Muir, Preston, Leschnault, Chain Avenue, Moodiarup, Tweed River, Tweed Road, Muntadgin, Wickepin and Fitzgerald River have been identified by taxonomists, and herbarium specimens for each are lodged at the Western Australian Herbarium (PERTH). Genotypes of individuals from the 14 reference populations were utilised to construct the reference data set. We aimed to genotype 48 individuals from each of the four subspecies although available sample numbers varied and 48, 43, 45 and 48 individuals were genotyped from subsp. *saligna*, *stolonifera*, *pruinescens* and *lindleyi* respectively.

The utility of the diagnostic tool created using the reference populations was assessed using individuals collected from six other test populations. The test populations were chosen from a number of populations where accurate subspecies identification is desired and where morphological assessment in the field was difficult. Three of the populations are planted. The Toodyay population is a planted stand on shire land where the seed source is unknown. It has been identified morphologically as subsp. *saligna* and the population has been used in studies on inter-subspecific gene flow (Millar et al. 2008a; Millar et al. 2008b). The populations labelled Dongara and Northampton were collected from a provenance trial and the seed for this trial was collected from these

areas in the northern range of the distribution of subsp. *lindleyi*. The remaining populations are from natural stands. The Hesters population was identified as subsp. *stolonifera* by both morphological and previous genetic analysis (George et al. 2006), although introduced plants of subsp. *saligna* occur nearby. The Weinup population has been used as a provenance seed collection site representative of subsp. *pruinescens*. The Bandy Creek population was originally identified morphologically as subsp. *lindleyi* although the genetic study of George et al. (2006) identified it as subsp. *saligna*. A new collection of plants from this population was used in this study to provide verification of its genetic status. For each of the test populations eight individuals were sampled except for the Dongara population where the seven trees present in the provenance trial were sampled. Details of the reference and test sites including geographic coordinates and herbarium sheet numbers are given in Table 6.1 and locations are illustrated in Figure 6.1.

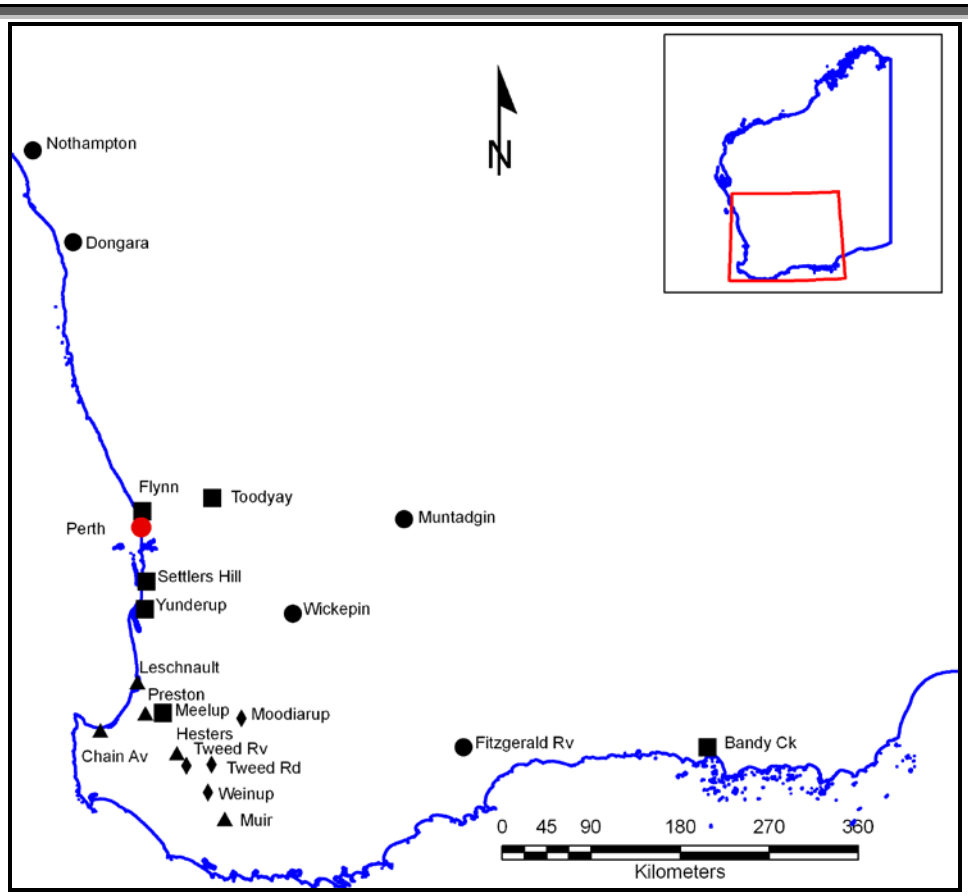

Figure 6.1 Geographic location of sampled populations of *Acacia saligna*. Population names refer to the populations in Table 6.1. ■ subspecies *saligna*, ▲ subspecies *stolonifera*, ♦ subspecies *pruinescens*, ● subspecies *lindleyi*.

Table 6.1 Details of sampled populations of *Acacia saligna* used as reference and test populations to demonstrate the use of the diagnostic genetic identification.

Population Name	Subspecies	Latitude E	Longitude S	Number of individuals sampled	WAHERB sheet number	Reference or Test population
Settlers Hill	*saligna*	32 19 25	115 48 18	14	6796265	R
Flynn	*saligna*	31 41 14	115 46 03	15	6796273	R
Yunderup	*saligna*	32 34 20	115 47 27	14	6451411	R
Meelup	*saligna*	33 35 48	126 04 56	5	6451446	R
Muir	*stolonifera*	34 30 16	116 38 13	15	6115233	R
Preston	*stolonifera*	33 31 43	115 58 12	14	06115276/ 06451403	R
Leschnault	*stolonifera*	33 13 04	115 41 36	14	6451438	R
Chain Avenue	*stolonifera*	33 40 55	115 16 57	5	6451454	R
Moodiarup	*pruinescens*	33 35 36	116 49 23	15	6183263	R
Tweed River	*pruinescens*	34 00 21	116 29 31	15	6183336	R
Tweed Road	*pruinescens*	34 00 06	116 13 08	15	6020208	R
Muntadgin	*lindleyi*	31 45 29	118 34 59	15	6183166	R
Wickepin	*lindleyi*	32 37 48	117 23 01	15	06183474/ 06183182	R
Fitzgerald River	*lindleyi*	33 49 43	119 15 45	18	6183255	R
Bandy Creek		33 45 55	121 55 52	8	06183220/ 06183212	T
Toodyay		31 34 20	116 30 57	8	-NA	T
Hesters		33 54 19	116 06 22	8	6020232	T
Weinup		34 15 47	116 27 25	8	7255144	T
Dongara		NA	NA	7	-NA	T
Northampton		28 23 10	114 40 47	8	-NA	T

Subspecies indicates the subspecies to which the population was assigned based on morphological analysis (McDonald MW and Maslin BR ms), WAHERB, Western Australian Herbarium; -NA data not available.

DNA extraction and microsatellite genotyping

For the reference populations DNA samples used in the previous study of George et al. (2006) were used. DNA was extracted from mature phyllodes, sampled from individuals in the test populations, except for Weinup, using a modified cetyltrimethyl ammonium bromide (CTAB) extraction method (Byrne et al. 1993) with the addition of 0.1 M sodium sulfite to the extraction and wash buffers (Byrne et al. 2001). For the Weinup population, DNA was extracted from seedlings that had grown to a height of approximately 5 cm, using a modified Doyle and Doyle procedure (Doyle and Doyle 1990).

Genotypes for all individuals were obtained using five microsatellite markers previously developed for use in *A. saligna* (Millar and Byrne 2007). Microsatellite loci As2.13, As2.17, As2.20, As2.47 and As2.61 were used. These five loci were identified as the

most informative for subspecies differentiation after initial screening of all ten primers on two individuals from each of the subspecies followed by further screening for alleles unique to subspecies in the reference populations. Genotypes of all reference and test samples were determined for the five selected microsatellite loci as described in Millar et al. (2007). The size of PCR products was determined by automated fluorescent scanning detection using an Applied Biosystems 3730 DNA Analyser (Applied Biosystems, Foster City CA, USA).

Determination of species structure

We used Bayesian model-based clustering as implemented in STRUCTURE 2.1 (Pritchard et al. 2000) to determine the structure within *A. saligna* and to assign individuals from reference populations to identified clusters. Most of the running parameters were set to default values as suggested in the users manual. Length of the burn in period was set to 10 000, number of Markov chain repetitions was set to 100 000 and 10 iterations were conducted for each number of clusters (K). We used a number of modelling approaches to determine the species structure.

Firstly, assignment of individuals in reference populations was tested to determine the most likely number of clusters. Prior population information was not used (i.e. the PopFlag element was set to 0). Analysis was initially conducted using a model allowing for the inference of admixture (alpha) and further analysis was conducted using the inferred value of alpha. K (number of clusters) was tested with values from one to eight with 10 iterations for each value of K. The most likely number of clusters was assessed using both values of Ln P(D) and the *ad hoc* quantity (ΔK) of Evanno et al. (2005), based on the second order rate of change of the likelihood function with respect to K.

As the aim of the study was to use the reference populations to form the basis of a diagnostic tool for subspecies identification, the assignment of individuals in reference populations was then conducted using prior information. The prior population information element was turned on (PopFlag = 1) and analysis was conducted using a model without admixture, for the most likely number of clusters (K) produced by the previous analysis. The average proportion of membership (Q) of the reference populations to the inferred clusters was assessed at the optimal K value.

The presence of first generation migrants in the reference populations was tested using GENECLASS2 (Piry et al. 2004). An L = L_home likelihood computation, which is the likelihood computed from the population where the individual was sampled, was used for migrant detection. A default frequency of 0.01 was used in case of a missing allele (Paetkau et al. 2004).

Multivariate principal component analyses was conducted for the reference samples using a covariance genetic distance matrix constructed by AMOVA (Analysis of Molecular Variation) in GENALEX 6 (Peakall and Smouse 2006). The matrix was then ordinated in a multidimensional space by principal coordinate analysis (PCA) using a standardised data set to detect genetically homogeneous grouping of reference populations.

Assignment of Test Individuals

To test the assignment of individuals from the test populations to clusters created using the reference data set analysis was conducted with STRUCTURE 2.1 (Pritchard et al. 2000) using both the reference and test data sets combined. Analysis was conducted using prior population information for individuals from the reference data set (PopFlag = 1), and no prior information (PopFlag = 0) for individuals from test populations using a model with no admixture. The number of clusters (K) was set to four. In this way, we used STRUCTURE 2.1 to estimate the posterior probability that each test individual belongs to a certain cluster corresponding to each of the proposed subspecies of *A. saligna* (q_i).

Population Genetic Parameters

Population genetic parameters for the subspecies based on the reference populations were calculated using GDA (Lewis and Zaykin 2001). Pairwise comparisons between the populations of each subspecies were conducted for these parameters using a T-test with 95% confidence intervals in SPSS (SPSS 2001).

Deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium were tested for all loci using GENEPOP (Raymond and Rousset 1995). The overall inbreeding coefficient (F), coancestory coefficient (θ) and degree of inbreeding within subspecies (f) (Weir and Cockerham 1984) and Nei's total genetic diversity (H_T) (Nei 1987) were

calculated for reference populations using FSTAT Version 2.9.3.2 (Goudet 2001). The 95% confidence interval was calculated by bootstrapping over loci 1000 times.

Results

Determination of species structure

The reference data set was used for analysis of population structure to test for the optimum number of clusters. Genetic structure in the reference data set was optimally partitioned into four groups (Figure 6.2a). Values of Ln P(D) began to plateau evenly at K = 4, however, Ln P(D) values continued to increase slightly for increasing K (Figure 6.3a), so the *ad hoc* quantity (ΔK) of Evanno et al. (2005) was investigated and showed a clear mode at K = 4 (Figure 6.3b). The mean standard error of the variance in Ln P(D) over all 10 iterations for K = 4 was 2.91. Each cluster was comprised of reference populations from one of the four proposed subspecies and values of average proportion of membership (Q) of pre-defined reference populations to clusters corresponding to their proposed subspecies were high (Table 6.2).

Individuals from reference populations identified morphologically as subsp. *stolonifera* formed a distinct cluster (cluster 2) and individuals from reference populations identified as subsp. *lindleyi* formed another distinct cluster (cluster 4). Individuals of these subspecies were assigned to their putative subspecies with assignment probabilities (q_i) of greater than 90%.

The majority of individuals from reference populations of subsp. *saligna* were placed in a separate cluster (cluster 1) and all but one individual from reference populations of subsp. *pruinescens* formed another distinct cluster (cluster 3). A level of admixture was evident for individuals from populations of both these subspecies. Assignment probabilities of 19 individuals from reference populations identified morphologically as subsp. *saligna* were greater than 50% for cluster 3 (subsp. *pruinescens*) and one individual from a reference population identified morphologically as subsp. *pruinescens* showed mixed genetic affinity for cluster 1, cluster 2 and cluster 3. Overall, the probability of individuals from reference populations of subsp. *saligna* being assigned to cluster 1 was 60%. Individuals from reference populations identified as subsp. *pruinescens* were assigned to cluster 3 with 90.5% probability (Table 6.2).

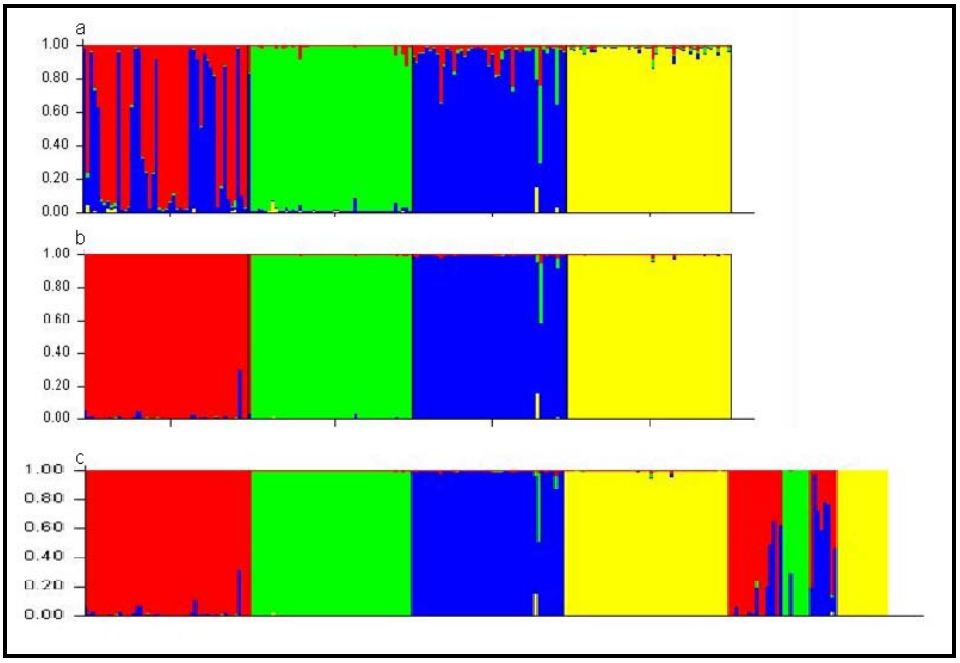

Figure 6.2 Bar plots representing the identity of individuals of *Acacia saligna* based on assignment using Bayesian modelling. Each individual is represented as a vertical line partitioned into K coloured segments whose length is proportional to the individual coefficients of membership in the K clusters that represent subspecies of *A. saligna*. a: reference populations using an admixture model with alpha = 0.032 for K = 4, b: reference populations using prior population information and a model without admixture for K = 4, c: reference and test populations combined using a model without admixture for K = 4. Cluster 1 (red): subsp. *saligna*, cluster 2 (green): subsp. *stolonifera*, cluster 3 (blue): subsp. *pruinescens*, cluster 4 (yellow): subsp. *lindleyi*.

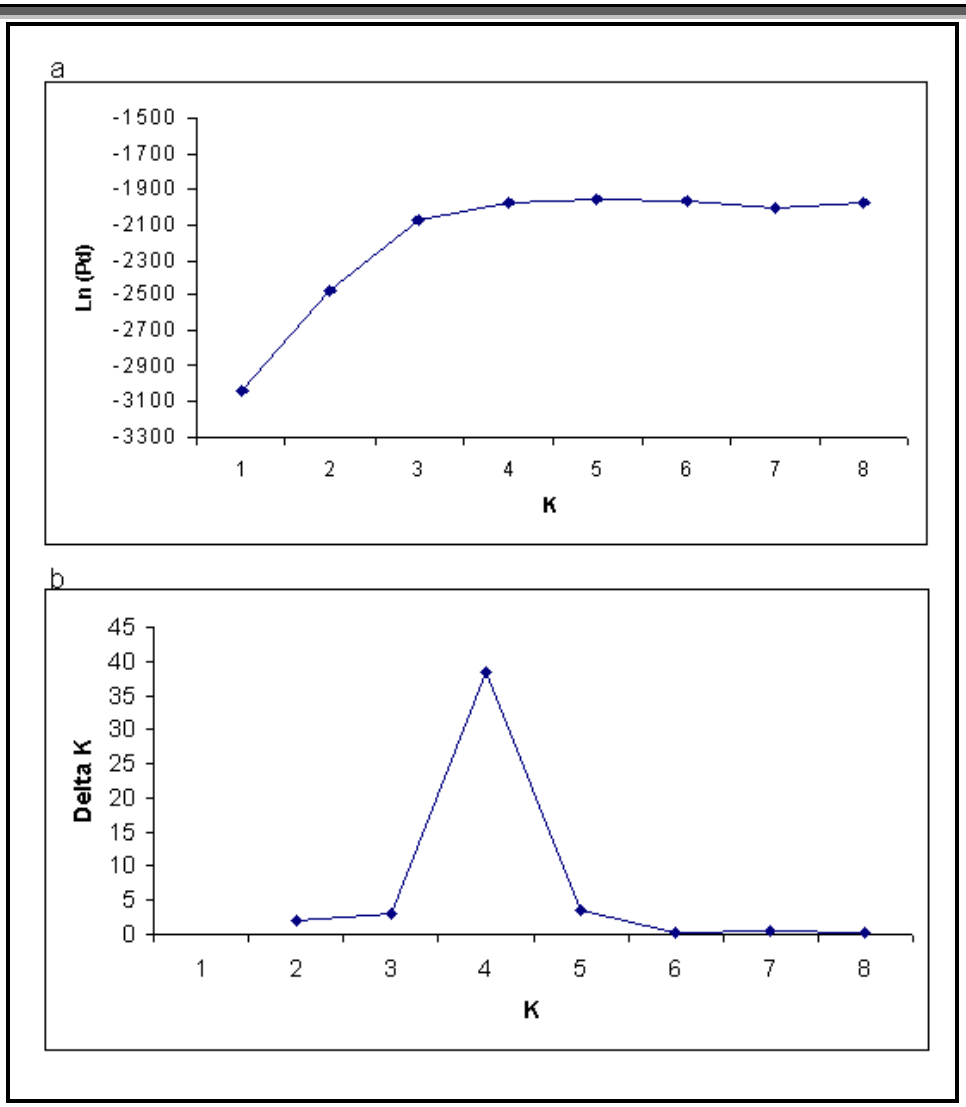

Figure 6.3 Identification of optimal number of clusters in Bayesian assignment analysis of reference populations of *Acacia saligna*. a: Increase of Ln P(D) against the number of K clusters obtained for K = 1-8 and b: D. ΔK computed after Evanno et al. (2005) against the number of clusters for K = 1-8 for populations in the reference data set.

Table 6.2 Values of average proportion of membership (Q) of pre-defined reference populations in four inferred clusters of *Acacia saligna*.

	Subspecies	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Without prior population information	*saligna*	**0.603**	0.008	0.381	0.008
	stolonifera	0.016	**0.949**	0.027	0.007
	pruinescens	0.055	0.032	0**.905**	0.009
	lindleyi	0.011	0.009	0.009	**0.971**
With prior population information	*saligna*	**0.984**	0.001	0.015	0.001
	stolonifera	0.022	**0.975**	0.002	0.001
	pruinescens	0.006	0.011	**0.979**	0.004
	lindleyi	0.002	0.001	0.001	**0.996**

Membership was computed using STRUCTURE 2.1 both without and with prior population information, under a model allowing for admixture using five microsatellite loci.

The highest Q values are indicated in bold for each cluster.

One individual from the Muir reference population of subsp. *stolonifera* was assigned with 99.1% probability to cluster 1, corresponding to the subsp. *saligna* genetic group. This individual was identified using GENECLASS2 as a first generation migrant from subsp. *saligna* into the Muir population. As the Muir population occurs on a highway roadside and subsp. *saligna* is often used for roadside revegetation programs it is not unlikely that this individual was indeed a migrant that was incorrectly sampled as part of the Muir population. For this reason, this individual was removed from further analysis involving the assignment of individuals from test populations.

Results of multivariate principal component analysis illustrate the high degree of genetic divergence between subsp. *stolonifera* and the other three subspecies, and between subsp. *lindleyi* and the other three subspecies for populations in the reference data set (Figure 6.4). It also illustrates the high level of genetic affinity between populations of subsp. *saligna* and subsp. *pruinescens*. The first axis separated populations of subsp. *stolonifera* and *lindleyi* from subsp. *saligna* and *pruinescens*. The second axis separated subsp. *stolonifera* from subsp. *lindleyi* but did not separate subsp. *saligna* from subsp. *pruinescens*. The first and second component explained 39.80% and 27.71% of the variation in the reference sample data set respectively.

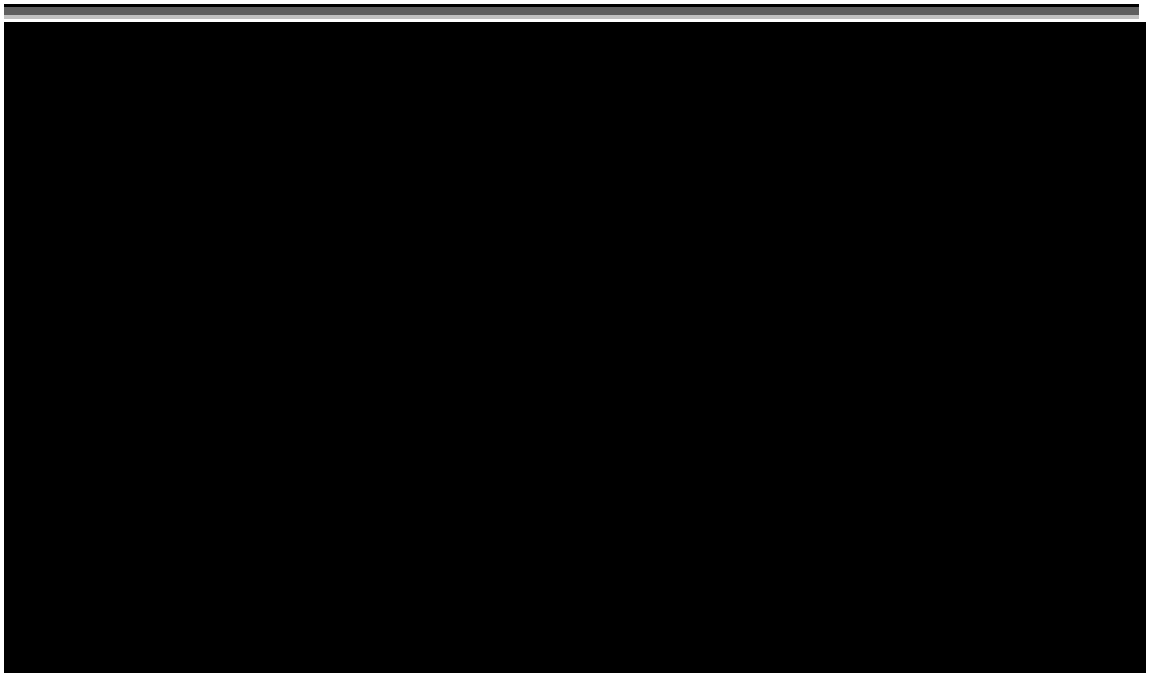

Figure 6.4 Principal component analysis of genotypic data using five microsatellite loci for individuals in the reference populations of *Acacia saligna*.

Because the reference data set is to be used as a diagnostic tool designed for the assignment of unknown individuals to one of the four subspecies of *A. saligna,* Bayesian assignment analysis was then conducted using prior population information for a model without admixture and K = 4. Under this model, all reference individuals clustered into four groups as described previously with improved resolution, as shown by the values of the average proportion of membership of pre-defined reference populations to the four inferred clusters (Q > 97%, Table 6.2). Using prior information improved the probability of the assignment of subsp. *saligna* individuals from 60% to 98%. Individual assignments to each cluster are illustrated in Figure 6.2b.

Test Population Assignment

Analysis of genetic affinities when test populations were included with the reference data set allowed for the assignment of all test individuals to one of the four genetic clusters (Figure 6.2c). Values of the average proportion of membership of all test populations to the four inferred clusters, corresponding to the four proposed subspecies of *A. saligna*, are given in Table 6.3.

Table 6.3 Values of average proportion of membership (Q) of test populations in four inferred clusters computed using STRUCTURE 2.1, under a model not allowing for admixture with five microsatellite loci.

Population	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Bandy Creek	0.987	0.000	0.0131	0.000
Toodyay	0.723	0.006	0.271	0.000
Hesters	0.000	0.961	0.0384	0.000
Weinup	0.427	0.003	0.565	0.005
Dongara	0.000	0.000	0.000	1.000
Northampton	0.000	0.000	0.000	1.000

The highest Q values are indicated in bold for each cluster.

All individuals from Bandy Creek were assigned to subsp. *saligna* with probabilities greater than 92%. Five individuals from the Toodyay population were assigned to subsp. *saligna* at probabilities greater than 75%. The remaining three Toodyay individuals showed genetic affinity with subsp. *pruinescens* at q_i = 44.5%, 61.8% and 65.4%. Seven of the eight individuals from the Hesters population were assigned with 100% probability to subsp. *stolonifera*. The remaining Hesters individual showed 30.7% affinity with subsp. *pruinescens*. The Weinup population showed mixed affinity for subsp. *pruinescens* and subsp. *saligna*. Four individuals were assigned to subsp. *pruinescens* at q_i values greater than 70% and one individual at a probability greater than 50%. The remaining three individuals were assigned at probabilities greater than 50% to subsp. *saligna*. All individuals from the Dongara and Northampton populations were assigned to subsp. *lindleyi* with 100% probability.

Population Genetic Parameters

Population analysis of nuclear microsatellite variation using five loci revealed moderate to high allelic diversity for the reference populations (Table 6.4). From 11 to 30 alleles per locus were detected with the highest number of alleles in any one population being 30. Means obtained over all populations gave 89% polymorphic loci (*P*), 3.57 alleles per locus (*A*) and 3.86 alleles per polymorphic locus (A_p) (Table 6.4). Observed heterozygosity (H_o) averaged 0.427 and expected heterozygosity (H_e), averaged 0.476 over all populations. Mean values for each parameter were greatest for subsp. *lindleyi* although pairwise comparisons between the subspecies showed that the difference was not significant at the 95% confidence level.

Table 6.4 Population genetic parameters of five microsatellite loci used to genotype individuals of *Acacia saligna* reference populations.

Subspecies	Population Name	P	A	A_p	H_e	H_o	F_{IS}
saligna	Settlers Hill	0.80	3.60	4.25	0.448	0.323	0.287
	Flynn	0.80	3.60	4.25	0.447	0.347	0.230
	Yunderup	0.80	3.00	3.50	0.466	0.371	0.210
	Meelup	1.00	3.80	3.80	0.613	0.400	0.375
	Bandy Creek	0.60	2.40	3.33	0.290	0.200	0.325
	Toodyay	1.00	3.00	3.00	0.530	0.325	0.403
	Mean	0.83	3.23	3.69	0.465	0.327	0.305
stolonifera	Hesters	1.00	3.80	3.80	0.635	0.625	0.016
	Muir	1.00	4.40	4.40	0.582	0.357	0.395
	Preston	1.00	3.00	3.00	0.328	0.343	-0.047
	Leschnault	0.60	2.20	3.00	0.204	0.227	-0.012
	Chain Avenue	1.00	3.00	3.00	0.413	0.460	-0.132
	Mean	0.92	3.28	3.44	0.434	0.402	0.044
pruinescens	Weinup	1.00	3.80	3.80	0.464	0.440	0.053
	Moodiarup	0.80	3.40	4.00	0.456	0.383	0.169
	Tweed River	1.00	2.80	2.80	0.420	0.387	0.083
	Tweed Road	0.80	3.00	3.50	0.354	0.360	-0.018
	Mean	0.90	3.25	3.53	0.424	0.393	0.072
lindleyi	Muntadgin	1.00	4.40	4.40	0.618	0.627	-0.014
	Wickepin	1.00	6.00	6.00	0.585	0.548	0.065
	Fitzgerald River	1.00	5.60	5.60	0.617	0.597	0.035
	Dongara	0.80	3.20	3.75	0.523	0.628	-0.222
	Northampton	0.80	3.40	4.00	0.523	0.600	-0.016
	Mean	0.92	4.52	4.75	0.573	0.600	-0.030
	Overall mean	0.89	3.57	3.86	0.476	0.427	0.107
	Overall means for RFLP's	0.64	2.65	3.57	0.282	0.278	0.030

Population names refer to those in Table 6.1.

For comparison overall mean values for RFLP markers are provided from George et al. (2006).

P: mean proportion of polymorphic loci, A: mean number of alleles per locus, A_p: mean number of polymorphic alleles per locus, H_e: expected heterozygosity, H_o: observed heterozygosity, F_{IS}: Wright's inbreeding coefficient.

Deviations from Hardy-Weinberg equilibrium were detected ($p < 0.01$) for the Muir population at loci As2.20 and As2.47, for the Tweed Road population at locus As2.61 and for the Settlers Hill, Flynn, Yunderup, Meelup, Bandy Creek, Muir and Leschnault

populations at locus As2.17. No significant linkage disequilibrium was detected among loci (p = 0.01).

Total genetic diversity (H_T) was high overall and high for populations of each subspecies combined, ranging from 0.454 for subsp. *pruinescens* to 0.621 for subsp. *lindleyi* (Table 6.5). Population differentiation was high for the species overall (θ = 0.355) indicating strong intraspecific structuring. Conversely, population differentiation values were low within each of the four subspecies indicating similarity of populations within subspecies. Estimates of inbreeding including Wright's inbreeding coefficient (F_{IS}, Table 6.4), the overall inbreeding coefficient (F) and degree of inbreeding within subspecies (f, Table 6.5) were greatest for subsp. *saligna* and lowest for subsp. *lindleyi*.

Table 6.5. Gene diversity and inbreeding coefficients for reference samples of subspecies of *Acacia saligna*. For comparison, overall mean values for RFLP markers are provided from George et al. (2006).

Subspecies	F	θ	f	H_T
saligna	0.375 (0.150, 0.680)	0.125 (0.051, 0.181)	0.286 (0.047, 0.650)	0.535
stolonifera	0.253 (0.068, 0.383)	0.158 (0.098, 0.247)	0.112 (-0.051, 0.206)	0.498
pruinescens	0.218 (0.019, 0.333)	0.075 (0.024, 0.174)	0.058 (-0.032, 0.196)	0.454
lindleyi	0.055 (0.001, 0.089)	0.069 (0.044, 0.094)	-0.015 (-0.057, 0.017)	0.621
Overall mean	0.423 (0.303-0.565)	0.355 (0.241-0.540)	0.105 (0.012-0.205)	0.729
Overall mean for RFLP's	0.392 (0.314, 0.473)	0.349 (0.263, 0.441)	0.066 (0.002, 0.120)	0.417

F inbreeding coefficient, θ co-ancestry coefficient, f degree of inbreeding within subspecies, H_T total genetic diversity.
95% bootstrapping confidence interval in parentheses.

Discussion

In this study we demonstrated the use of Bayesian analysis implemented in STRUCTURE 2.1 (Falush et al. 2003) for the assignment of individuals of the *A. saligna* species complex to one of four main proposed subspecies. This 'diagnostic' tool is required as the subspecific taxonomic characterisation of populations of *A. saligna* in the field is often difficult because the diagnostic morphological characters are only present during flowering.

Multilocus genotype data from only a few informative microsatellite markers was initially used with no prior population information for a reference data set to provide an estimate of the number of genotypic clusters and partitioning among reference populations. The optimal number and partitioning of clusters showed clear alignment to known morphological characterisations of the reference populations and indicated four clusters corresponding to the four main proposed subspecies. Values for the average proportion of membership of pre-defined reference populations of subsp. *pruinescens*, *stolonifera* and *lindleyi* were greater than 90%, while that of subsp. *saligna* was 60%. The addition of prior population knowledge improved the average proportion of membership of pre-defined reference populations to extremely high probabilities (Q > 97.5%) for each of the four subspecies. These results illustrate the strong resolution of genetic structuring possible using Bayesian statistical analysis with data from a few informative microsatellite markers in the presence of prior population information. Much greater resolution between populations of subsp. *saligna* and subsp. *pruinescens* was achieved using this approach than with multivariate principal component analysis.

Populations of subsp. *saligna* and subsp. *pruinescens* in the reference data set showed a degree of genetic affinity under a model allowing for population admixture without prior population information. Resolution of individuals and populations of each of the two subspecies was greatly improved over that obtained using RFLP data, where the two subspecies could not be genetically differentiated (George et al. 2006), however, these two subspecies show differences in morphological characteristics in the field, have disjunct natural distributions and there are no reports of hybridisation between natural populations. The closest populations studied here are separated by distances of more than 120 km and reference populations were selected on the basis of being comprised of individuals of only one subspecies. These characteristics make it unlikely that the levels of admixture found for populations of these two proposed subspecies are the result of sampling error or mixed populations where substantial levels of gene flow and hybridisation may be occurring. Rather, it is more likely the levels of admixture are indicative of a high degree of natural genetic affinity.

Using microsatellite data and Bayesian analysis, we have achieved an adequate level of resolution between the subsp. *saligna* and subsp. *pruinescens* and the results presented here do support the presence of four genetic lineages within the species complex. The

improved resolution obtained using microsatellite loci is most likely due to the greater level of genetic diversity detected with these markers compared to RFLPs. The five loci used here showed higher levels of diversity than the 23 RFLP loci used by George et al. (2006) for all population parameters (number of alleles, polymorphic loci, heterozygosity) and for total gene diversity. Both markers detected a similar level of genetic differentiation within the species. While there is some bias in this comparison as the loci used here were selected for their diversity, it is common for microsatellite loci to detect greater levels of diversity than other markers. In comparisons for several eucalypt species, diversity in microsatellite loci has been found to be twice as variable as RFLP loci and on average four times more variable than isozyme loci (Byrne 2007).

By providing prior population information and using the data from reference populations as a diagnostic tool, it was possible to assign individuals from test populations to a genetic cluster corresponding to one of the four main subspecies. The majority of individuals from test populations were assigned to one of the four subspecies with a high degree of confidence. In particular, individuals from a natural population identified as subsp. *stolonifera* (Hesters) and individuals from two populations of subsp. *lindleyi* sampled from a provenance trial (Dongara and Northampton) were unequivocally assigned to genetic groups according to their putative morphological identification.

The identity of individuals from test populations of subsp. *saligna* and *pruinescens* was not as well resolved as that of subsp. *lindleyi* and *stolonifer*a. Individuals from the planted population at Toodyay, believed to be subsp. *saligna*, and the natural population at Weinup, believed to be subsp. *pruinescens*, were assigned to either subsp. *saligna* or *pruinescens*, with a degree of mixed genetic affinity observed for some individuals. The Toodyay population is comprised of trees from seed of unknown origin and confirmation of the taxonomic characterisation of the stand was desired, as it has been used in studies on intra-subspecific gene flow within the *A. saligna* species complex (Millar et al. 2008a; Millar et al. 2008b). There may be two explanations for the level of mixed genetic affinity observed in the Toodyay individuals. Firstly, analysis of genetic structuring in the *A. saligna* species complex has shown genetic affinities between subsp. *saligna* and subsp. *pruinescens* that is not fully resolved by the microsatellite markers used here. The original seed source for the planted stand is

unrecorded and may have originated from a population with genetic affinity to each subspecies. Secondly, the seed collection used to establish the planted stand may have come from a bulk seed collection sourced from a number of populations including populations of both subsp. *saligna* and subsp. *pruinescens* as well as populations with some level of genetic admixture. Alternatively, the seed may have been sourced from another planted stand with mixed germplasm. The admixture identified in a few of the individuals at the Weinup population of subsp. *pruinescens* most likely reflects the higher genetic affinity of the two subspecies, given the strong assignment of the majority of individuals to subsp. *pruinescens*.

Assignment analysis of the Bandy Creek population provided clarification of its taxonomic identity. This population had originally been identified as being the 'Typical' variant (subsp. *lindleyi*) in a field survey and was included in the genetic study of George et al. (2006). However, the population was shown to have strong genetic affinity with the subspecies *saligna*/*pruinescens* cluster and the authors had no explanation for the anomalous assignment. Repeat analysis with resampling confirmed the original genetic results of George et al. Subsequent morphological investigation of the population, and others along the coast to the east, have led to the identification of previously unknown populations of subsp. *saligna* in this area. The results presented here clarify that it was the original taxonomic characterisation of the Bandy Creek population based on morphological characteristics and location that was anomalous, not the genetic affinity.

Conclusions

The accurate identification of populations and their constituent individuals in species complexes, such as that of *Acacia saligna*, is essential although often difficult to achieve. Morphological characteristics show great variation even within each subspecies and the main morphological characteristics that form the basis of subspecies identification are only observable at certain times of the year and at certain developmental stages, and may be further hampered by phenotypic variation influenced by the specific growing environment. To date, the difficulties with accurate identification of the different informal subspecies of *A. saligna* has hampered determination of the geographic distributions of each subspecies, led to uncertainties in

the sampling of genetic material for the establishment of a breeding program for the species domestication, and resulted in lack of knowledge regarding the *in situ* conservation of populations.

The results of this study showed the clear determination of four genetic groups that are significantly differentiated, and correspond to each of the four proposed main subspecies in *A. saligna*. The PCR based diagnostic approach we have developed in this study provides an accurate tool to characterise populations of the four main subspecies. It is envisaged that this tool will be widely applicable to the identification of populations of unknown origin, especially naturalised and invasive populations existing outside the species natural range, such as those in South Australia and in South Africa.

Acknowledgements

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Chapter Seven
General Discussion

The aim of this research was to develop the knowledge base for an effective domestication and breeding program and risk management plan for the use of the *Acacia saligna* species complex as an agroforestry crop for the low rainfall areas of southern Australia. The overall objectives of this research were to:

- Investigate the mating system, patterns of pollen dispersal and level and distance of inter-subspecific pollen immigration from natural populations that can be expected in seed production stands of *Acacia saligna*. The knowledge generated here informs the domestication and breeding program for the efficient management of seed production stands in which genetic gain is maximised and genetic diversity maintained in seed crops.

- Investigate patterns of genetic contamination via pollen flow from planted stands that can be expected in natural populations of *Acacia saligna*. With this knowledge, agroforestry populations of *Acacia saligna* can be managed to ensure that inter-subspecific pollen immigration into remnant populations from populations planted for agroforestry is minimised.

- To investigate genetic structuring present within *A. saligna* and assess the congruence with taxonomic reclassification of the species complex.

- To produce a diagnostic tool for the rapid and accurate identification of individuals and populations of *A. saligna* at the subspecies level.

These objectives were achieved and the principal findings of the research were:

- Analysis of the mating system in a planted stand of *A. saligna* subsp. *saligna* indicated a high level of outcrossing, with a multilocus outcrossing rate of 0.98, and little true inbreeding. Analysis of pollen dispersal patterns indicated an essentially random pattern of mating within the stand. Although an excess of mating at short distances was observed, pollen dispersal was detected across the

whole stand, there was heterogeneity in pollen clouds experienced by maternal trees, and over half of trees within the stand contributed to the observed paternal pollen pools. Inter-subspecific pollen immigration into the planted stand of subsp. *saligna* from trees in remnant populations of subsp. *lindleyi* was detected in 14% of progeny analysed and occurred over large distances greater than 1500 m.

- Analysis of pollen-mediated gene flow between remnant patches of *A. saligna* subsp. *lindleyi* revealed that extensive intra-subspecific gene flow is maintained in the fragmented agricultural landscape of Western Australia. Intra-subspecific gene flow between remnant patches was detected over distances greater than 1500 m. Additionally, extensive levels of inter-subspecific pollen-mediated gene flow from genetically divergent populations may pose a threat to remnant populations of *A. saligna*. Inter-subspecific pollen immigration (genetic contamination) into remnant populations of subsp. *lindleyi* from trees in a planted stand of subsp. *saligna* was detected in 32% of progeny analysed and also occurred over large distances greater than 1500 m.

- High levels of genetic sub-structuring congruent with the current taxonomic revision were detected within the *A. saligna* species complex. Adequate resolution between subspecies *saligna* and subspecies *pruinescens* was achieved and the elevation of these two entities to subspecific rank is supported.

- Highly informative microsatellite markers proved suitable for use as a rapid diagnostic tool for the accurate identification of individuals and populations of *A. saligna* at the subspecies level. A PCR based diagnostic tool was developed and used to characterise populations into one of the four main proposed subspecies of *A. saligna* with high probability.

Principal significance of the findings

These findings have a number of outcomes of significance for the domestication and breeding program, for the conservation of natural remnant populations, and for further

research in the *A. saligna* species complex. The following discussion explores the significance of the findings for these areas of research.

Domestication and breeding

Improvement of the *A. saligna* species complex for utilisation as a commercial agroforestry crop is likely to involve planting a number of seed production stands. The focus of domestication and breeding in Australia will involve selection and breeding for traits such as survival, improved form and biomass production, and improved fodder quality. Because of the high levels of genetic diversity found in the species, it is likely that the initial approach in the breeding program will involve provenance selection for superior genotypes that exhibit these agronomically important traits. Superior provenances may then be utilised as early commercial varieties and early provenance trials converted to seed production stands by the culling or rouging of inferior genotypes. It is unlikely that the original sources of germplasm (natural populations) will produce commercial quantities of seed and, as the domestication program progresses, selective breeding may be employed to enhance agronomic traits and genetically improved seed will need to be bulked in seed production stands such as seed orchards.

Open pollinated seed orchards, managed for the rapid production of easily harvestable seed of improved genetic value, should be an efficient means of producing large quantities of improved seed of *A. saligna*. Analysis of the mating system and patterns of pollen dispersal in a planted stand of *A. saligna* subspecies *saligna* revealed a level of outcrossing equal to or higher than that found for several natural populations (George 2005) and an essentially random pattern of mating indicating that it should be possible to maintain high levels of outcrossing with minimal inbreeding in seed production stands and that mating patterns in seed production stands should approach panmixia. Seed production stands will be managed to minimise the level of inbreeding and maximise panmictic mating patterns as this ensures the maintenance of high levels of genetic diversity in resultant seed crops (Friedman and Adams 1985).

Seed production stands must also be managed to minimise the immigration of foreign pollen into stands, as pollen-mediated gene flow from trees outside the stand will act to dilute superior genotypes in the resultant seed crops. The extensive levels of both inter-

and intra-subspecific pollen-mediated gene flow, occurring over distances greater than 1500 m (Millar et al. 2008a; Millar et al. 2008b) indicate that seed production stands of *A. saligna* may be at significant risk of pollen contamination from trees outside the stand. One obvious management option to minimise gene flow is planting seed orchards outside the species natural distribution. However, the target areas for revegetation occur across the species natural distribution in Western Australia where any early provenance trials converted to seed orchards will be located. It is recommended that when seed production stands are planted inside the species natural range management options such as the use of barrier or guard rows or isolation by distance be used. It is also recommended that seed crops from production stands be monitored for genetic contamination to ensure that management practices aimed at minimising genetic contamination are effective.

Environmentally sustainable management

The highly outcrossed mating system and extensive levels of both inter- and intra-subspecific pollen-mediated gene flow detected in *A. saligna* also suggest that pollen-mediated gene flow from large stands of *A. saligna* planted for agroforestry will pose a threat to nearby remnant natural populations via genetic contamination. This is a pertinent issue for the conservation of small remnant populations in the highly disturbed and fragmented agricultural areas of south Western Australia.

This research detected a significant level of inter-subspecific gene flow (32%) into remnant populations of subspecies *lindleyi* from a planted stand of subspecies *saligna* and inter-subspecific gene flow occurring over distances greater than 1500 m. Data is presented for a single seed cohort for only one site and as temporal and spatial variation in levels and distances of gene flow are bound to occur, the results presented here may not be strictly applicable to this site each year nor broadly applicable at all other sites. The application of these results would benefit greatly from experimental replication for another seed cohort and/or at another site. The results however do indicate a high likelihood that levels of genetic contamination in nearby remnant populations from planted stands will be significant for *A. saligna*. Indeed levels of genetic contamination in remnant populations may be significantly greater than those found for this study site, as stands planted for improved seed production and those used for agroforestry purposes are likely to be much larger than the planted stand at this study site. It is perhaps worth

mentioning that the study site used in this research was chosen as it was the only site found after extensive searching where a compact obviously planted stand of subspecies *saligna* of suitable size to allow genotyping of all trees was found planted in close proximity to naturally occurring subspecies *lindleyi* trees.

It is desirable to assess and limit the levels of inter-subspecific gene flow into remnant populations of *A. saligna* from planted stands of genetically divergent germplasm as a precautionary step in the environmentally sustainable management of the crop for agroforestry. The precautionary principle must be applied as the impacts of genetic contamination in remnant populations are largely unknown and require investigation.

A range of management options aimed at reducing the risk of pollen-mediated genetic contamination into remnant populations from stands planted as agroforestry crops or for seed production do exist (Potts et al. 2003), although each will have to be assessed for efficacy. These options include the planting of barrier or guard rows around planted stands and the use of isolation distances between planted stands and remnant populations. Further options suitable for agroforestry crops but not seed production stands (which must flower and set seed) include the selection of genotypes with altered (significantly later or earlier) floral phenologies, the selection or breeding of infertile genotypes or genotypes of significantly reduced floral fecundity, harvesting crops before flowering commences, and the genetic regulation of floral development. The simplest option initially, will be ensuring that planted stands and remnant populations are sufficiently isolated, and isolation distances greater than 1500 m are recommended between seed production stands and natural populations. This approach will minimise both pollen-mediated gene flow into remnant populations from stands planted for agroforestry and from natural populations into stands planted for seed production.

Genetic relationships and subspecies identification

The *Acacia saligna* species complex, is currently described by four main proposed subspecies, that display a high degree of variation for biological, ecological and morphological traits, although populations are often cryptic in terms of morphological characteristics used to identify taxonomic entities (Byrne and Millar 2004; Maslin et al. 2006). The genetic relationships between subspecies have also not been fully resolved. The findings of this work confirmed high levels of genetic divergence between the four

proposed subspecies of *A. saligna* using genetic data obtained with highly polymorphic microsatellite markers developed specifically for *A. saligna*. An overall mean co-ancestry coefficient of 0.355 (Millar et al. 2008c) indicates significant sub-structuring into four genetic groups that correspond to the known morphological characterisations.

Two of the subspecies, subsp. *saligna* and subsp. *pruinescens*, show a degree of genetic affinity leading to some ambiguity as to the taxonomic entities present within the species complex. In a previous study by George et al. (2006) using RFLP data subsp. *saligna* and subsp. *pruinescens* could not be genetically distinguished. In the work presented here, the use of highly polymorphic microsatellite markers combined with Bayesian clustering analysis provided stronger resolution between these two subspecies than obtained previously using RFLP data. Although a level of genetic affinity between these two subspecies was still evident, it is recommended based on the findings presented here, that the two proposed subspecies should be elevated to subspecies rank, a result consistent with a current taxonomic revision of the species complex.

The genetic resolution of discrete entities within the *A. saligna* species complex enabled the development of a diagnostic tool to accurately identify populations at the subspecific level. Characterising natural populations is a first step for any domestication and breeding program and for conservation activities, and will also assist in the accurate characterisation of invasive populations outside of the species natural distribution both in Australia and overseas. Effective domestication and breeding programs require the collection of genetically diverse germplasm, which is achieved by ensuring seed is collected from natural populations over the entire range of each subspecies. Conservation efforts aimed at ensuring diversity is conserved at the genetic, subspecific and species levels require knowledge on the extent of the natural range of subspecific entities. To date, the accurate identification of populations of *A. saligna*, and hence knowledge of natural distributions at the subspecific level, has been hampered by the cryptic morphological variation present within the species complex. Major morphological traits on which the different subspecies are characterised are only present during floral development (McDonald and Maslin in prep) and this has led to difficulty in identifying populations in the field and a degree of ambiguity regarding the natural ranges of the subspecies.

The development of a PCR based diagnostic tool enables the accurate and rapid identification of natural populations at the subspecies level. Bayesian analysis of genotypic data obtained using highly polymorphic markers for a number of populations provides a tool that assigns individuals and hence, the populations they represent, to one of the four main subspecies of *A. saligna* with high probability. This diagnostic tool has immediate use in the identification of cryptic natural populations within the natural range of the species complex allowing the accurate delineation of the natural ranges of each subspecies.

The diagnostic tool may also find application in domestication programs already established overseas, reviewed by Midgley and Turnbull (2003), and in research into invasive populations outside the species' natural range. *Acacia saligna* has a long history of utilisation in a number of countries overseas. Domestication programs focussing on establishment, management and utilisation for animal fodder, human food, fuel wood, tannin production and erosion control have been conducted in many countries in North Africa, the Mediterranean, the Middle East and South America (Crompton 1992; Midgley and Turnbull 2003). While intensive breeding programs are not established (Midgley and Turnbull 2003), domestication programs require the accurate identification and characterisation of germplasm resources available for exploitation. The diagnostic tool for *A. saligna* may be put to use in assessing the origin and variability in terms of the subspecific characterisation of germplasm available in these domestication programs.

The *A. saligna* species complex also has a history of invasiveness where it has been planted in areas outside its natural range (Blood 2001; Maslin and McDonald 2004). The species is naturalised in all states in Australia, across the Mediterranean (Fox 1995; Muyt 2001) and is highly invasive in South Africa (Henderson 2001; Yelenick et al. 2004). Few of these populations have been identified at the subspecific level and it is not known if one or more subspecies are typically more invasive than others. The diagnostic tool developed here can be used to identify invasive populations at the subspecific level. This information can be used to assess whether more than one subspecies is invasive, whether multiple introductions of one or more subspecies have been made in certain areas, and whether there are any differences in the relative invasiveness of each subspecies.

Recommendations for future research

A number of areas of further research that will be valuable for efficient domestication and breeding programs and in the sustainable utilisation of *A. saligna* as an agroforestry crop have been identified. These are:

Assessment of management techniques for planted stands
The management options aimed at reducing the risk of pollen mediated genetic contamination into remnant populations from planted stands should be assessed for economic and practical viability and efficacy.

Management options that involve temporal isolation in flowering between natural and agroforestry population include the selection of genotypes with altered (significantly later or earlier) floral phenologies and the selection of infertile genotypes or genotypes of significantly reduced floral fecundity for agroforestry. Variation in floral phenology has not been formally assessed and is discussed further below. Variation in floral fecundity and female sterility has been observed in *A. saligna* (George 2005) and may allow trees displaying reduced flowering or sterility to be selected for in a breeding program. Sterile triploids have been found to occur naturally in some Acacia species including *A. aneura* (Miller et al. 2001) and *A. dealbata* (Blakesley et al. 2002). Sterile triploids have also been induced in the African acacia *A. nilotica* (Garg et al. 1996), and the technology is being developed in the plantation species *A. mangium*, *A. dealbata* and *A. mearnsii* to reduce the propensity of invasiveness in these species (ACIAR 2007). Information on the variation in phenology, fecundity and the occurrence of natural polyploids within *A. saligna* is lacking, and requires further investigation. However, selection or manipulation for genotypes displaying reduced fecundity or sterility is likely to be a long-term task. The genetic regulation of floral development, which is being investigated in *Eucalyptus globulus* (reviewed by Lemmetyinen and Sopanen 2004), is also an area of advanced research unlikely to produce rapid results employable on ground in the short term.

Another option to reduce the risk of pollen-mediated gene flow is to harvest agroforestry crops before flowering commences. As a short rotation crop this may be feasible in *A. saligna*, however, it should be noted that *A. saligna* can commence

flowering at 15 months (Fox 1995) and young trees of *A. saligna* with little biomass that would therefore be unsuitable for harvest are often observed flowering in the field (M. Millar pers obs). This option may therefore not be as viable for *A. saligna* as it may for longer lived species.

It is suggested that the efficacy of isolation distances between planted stands and remnant populations and of barrier or guard rows around planted stands should be the initial areas of research, because they are likely to be the most immediately deployable management options and are also likely to be economically viable.

Assessment of floral phenology

A formal assessment of the relative floral phenologies of each of the proposed subspecies of *A. saligna* is required for further assessment of the potential for pollen-mediated gene flow and hybridisation among the subspecies and for assessment of the potential use of temporal isolation as a management technique to minimise pollen-mediated gene flow into remnant populations and into seed production stands. The information could also be further exploited in the breeding program, for example, to improve the efficiency of controlled crosses and to maximise the amount of inter-subspecific gene flow within seed orchards. To gather this information it would be desirable to monitor the production and development of floral structures for a number of populations across the natural range of each of the subspecies, for a number of years. Floral development and phenology should also be assessed for different provenances of each of the subspecies at common sites to determine the relative influences of environment versus genetic regulation.

Assessment of hybridisation and introgression

Studies of natural and manipulated hybrids and knowledge of barriers to hybridisation, including pre-zygotic and post-zygotic barriers, are required to provide further information on the potential of hybridisation among the subspecies of *A. saligna* in intended planting areas (Potts et al. 2001). To gather this information it would be desirable to perform controlled crosses and test the hybrids under closely controlled field conditions (Tiedje et al. 1989). Unfortunately, controlled pollination is difficult for this species due to the nature of the inflorescences and floral development. The hermaphrodite flowers are small, grouped closely together in spherical inflorescences and stigmas are surrounded by a large number of pollen bearing anthers with little time

between stigma reception and anther dehiscence, making emasculation difficult (George 2005). Hybrid testing may also prove time consuming due to the long generation time of trees. Overcoming these difficulties should be a priority, as this information will also be desirable for advanced breeding programs where hybridisation followed by selection for progeny with improved characteristics is required.

Data will also be required on the long-term level and impact of hybridisation and introgression in populations where hybridisation is predicted to occur. Research should determine whether introgression is more likely to result in heterosis or outbreeding depression in hybrid progeny. Hybrid progeny will need to be assessed for a number of years and over a number of generations as the effects of hybridisation are often expressed at different stages of maturity and hybrid breakdown is often not expressed until the F2 generation (Hufford and Mazer 2003; Potts et al. 2003). Long term introgression of crop genes into wild relatives also has a history of producing or enhancing weediness traits in native populations, creating new weeds or enhancing of the effects of existing weeds (Ellstrand et al. 1999b). While it is unlikely populations of *A. saligna* used for agroforestry in the near future will possess strongly advantageous genes relative to natural populations, key traits influencing weediness may be transferred, such as the ability to propagate vegetatively via root suckering.

Assessment of invasive populations

The *A. saligna* species complex has a number of other biological traits giving it an extensive history of invasiveness when planted in areas outside its natural range. Assessments of invasive populations of *A. saligna* are required in order to determine whether certain subspecies or populations possessing specific biological characteristics have the propensity to be more invasive than others. The diagnostic tool developed here for the subspecific identification of populations can be used to identify invasive populations and the relative invasiveness of each subspecies and may provide information regarding characteristics that might make populations highly invasive. Recommendations can then be made as to the possible restricted use of certain subspecies outside their natural range.

Conclusion

Acacia saligna shows great potential as an agroforestry crop for the low rainfall agricultural areas of southern Australia that urgently require revegetation to combat increasing levels of salinisation. The work comprising this thesis provides valuable and timely information required for further domestication of the species and its environmentally sustainable management as an agroforestry crop.

Bibliography

ACF (2001) Australian land clearing, a global perspective: Latest facts and figures. Melbourne, Australian Conservation Foundation.

ACIAR. (2007) Development and evaluation of sterile triploids and polyploid breeding methodologies for commercial species of Acacia in Vietnam. Retrieved from: http://www.aciar.gov.au/project/FST/2003/002.

Adams W and J Burczyk (2000) Magnitude and implications of gene flow in gene conservation reserves. In: Forest conservation genetics: principles and practice. Eds A Young, D Boshier and T Boyle. Collingwood, Vic, CSIRO Publishing: 215-226.

Adams W, V Hipkins, J Burczyk and W Randall (1997) Pollen contamination trends in a maturing Douglas-fir seed orchard. Canadian Journal of Forest Research 27: 131-134.

ADSA (2001) Australian Dryland Salinity Assessment. Braddon, A.C.T., National Heritage Trust.

Armstrong T, R Fitzjohn, L Newstrom, A Wilton and G Lee (2005) Transgene escape: what potential for crop-wild hybridisation? Molecular Ecology 14: 2111-2123.

Arnold M (1992) Natural hybridisation as an evolutionary process. Annual Review of Ecology and Systematics 23: 237-261.

Atkins S and I Clark (2004) Fungal molecular diagnostics: a mini review. Journal of Applied Genetics 45: 3-15.

Austerlitz F, C W Dick, C Dutech, E K Klein, S Oddou-Muratorio, P E Smouse and V L Sork (2004) Using genetic markers to estimate the pollen dispersal curve. Molecular Ecology 13: 937-954.

Austerlitz F and P Smouse (2002) Two-generation analysis of pollen flow across a landscape.IV. Estimating the dispersal parameter. Genetics 161: 355-363.

Bacles C F E, J Burczyk, A J Lowe and R A Ennos (2005) Historical and contemporary mating patterns in remnant populations of the forest tree *Fraxinus Excelsior* L. Evolution 59: 979-990.

Barson M M, L A Randall and V Bordas (2000) Land cover change in Australia. Results of the collaborative Bureau of Rural Sciences - State agencies' project on remote sensing of land cover change. Canberra, Bureau of Rural Sciences.

Bartle J (1991) Tree crops for profit and land improvement. Western Australian Journal of Agriculture 32: 11-17.

Bartle J (2001) New perennial crops: mallee eucalypts, a model large-scale perennial crop for the wheatbelt. Outlook Conference 2001, National Convention Centre, Canberra, The Australian Bureau of Agricultural and Resource Economics, Canberra.

Bartle J, D Cooper, G Olsen and J Carslake (2002) *Acacia* species as large-scale crop plants in the Western Australian Wheatbelt. Conservation Science Western Australia 4: 96-108.

Bartle J and S Shea (2003) Development of mallee as a large-scale crop for the wheatbelt of W.A. Proceedings Australian Forest Growers 2002 National Conference. Private Forestry - Sustainable, Accountable and Profitable, Albany, Western Australia.

Bathgate A and D Pannel (2002) Economics of deep-rooted perennials in Western Australia. Agricultural Water Management 53: 117-132.

Bennell M, T Hobbs and M Ellis (2004) FloraSearch Species and Industry Evaluation – Low rainfall agroforestry options for southeastern Australia. RIRDC Report,. Adelaide, S.A. Department of Water, Land and Biodiversity Conservation.

Bennett A (1993) Fauna conservation in box and ironbark forests: a landscape approach. Victorian Naturalist 110: 15-23.

Bennett S J and J G Virtue (2003) Salinity mitigation versus weed risks - resolving conflicts of interest in introducing new plants. Scoping Study. CRC for Plant Based Management of Dryland Salinity.

Beresford Q, H Bekle, H Philips and J Mulcock (2001) The salinity crisis: Landscapes, communities and politics. Perth, Western Australia, The University of Western Australia Press.

Black A, K Forge and F Frost (2000) Extension and Advisory Strategies for Agroforestry. A report for the RIRDC/LWRRDC/FWPRDC Joint Venture Agroforestry Program. Barton, A.C.T., Rural Industries Research and Development Corporation.

Blakesley D, A Allen, T Pellny and A Roberts (2002) Natural and induced polyploidy in *Acacia dealbata* Link. and *Acacia mangium* Willd. Annals of Botany 90: 391-398.

Blood K (2001) Environmental weeds: A field guide for South Eastern Australia. Victoria, Australia, C. H. Jerram and Associates Science Publishers, CRC Weed Management Systems.

Broadhurst L, M Byrne, L Craven and B Lepschi (2004) Genetic congruence with new species boundaries in the *Melaleuca uncinata* complex (Myrtaceae). Australian Journal of Botany 52: 729-737.

Brown A and C Brubaker (2000) Genetics and the conservation and use of Australian wild relatives of crops. Australian Journal of Botany 48: 297-303.

Butcher P, S Decroocq, Y Gray and G Moran (2000) Development, inheritance and cross-species amplification of microsatellite markers from *Acacia mangium*. Theoretical and Applied Genetics 101: 1282-1290.

Butcher P, J Glaubitz and G Moran (1999) Applications for microsatellite markers in the domestication and conservation of forest trees. Forest Genetic Resources Information 27: 34-42.

Butcher P, G Moran and H Perkins (1998) RFLP diversity in the nuclear genome of *Acacia mangium*. Heredity 81: 205-211.

Byrne M (1999) High genetic identities between three oil mallee taxa, *Eucalyptus kochii* ssp. *kochii* ssp. *plenissima* and *E. horistes*, based on nuclear RFLP analysis. Heredity 82: 205-211.

Byrne M (2002) Phylogenetics and the conservation and utilisation of *Acacia* in Western Australia. Conservation Science Western Australia 4: 10-18.

Byrne M (2007) Phylogeny, diversity and evolution of eucalypts. In: Plant genome: Biodiversity and evolution 1, Vol 1E. Eds A K Sharma and A Sharma. New Delhi, India, Oxford and IBH Publishing: 303-346.

Byrne M and L Broadhurst (2002) Development of native germplasm for agroforestry. 12th Australasian Plant Breeding Conference, Perth, Western Australia, Australasian Plant Breeding Association Inc.

Byrne M and L Broadhurst (2003) Genetic diversity and the utilisation of *Acacia* species complexes in agroforestry in Western Australia. Australian Systematic Botany 16: 49-55.

Byrne M, C P Elliott, C Yates and D J Coates (2007) Maintenance of high pollen dispersal in *Eucalyptus wandoo*, a dominant tree of the fragmented agricultural region in Western Australia. Conservation Genetics doi: 10.1007/s10592-007-9311-5.

Byrne M, B Macdonald and M Francki (2001) Incorporation of sodium sulfite into extraction protocol minimizes degradation of *Acacia* DNA. BioTechniques 30: 742-748.

Byrne M and M A Millar (2004) Genetic systems and issues in the development of woody perennials for revegetation. Proceedings of the Conference Salinity Solutions: Working with Science and Society, Bendigo, Victoria, CRC for Plant-Based Management of Dryland Salinity.

Byrne M, G Moran and W Tibbits (1993) Restriction map and maternal inheritance of chloroplast DNA in *Eucalyptus nitens*. Heredity 84: 218-220.

Cagelli L and F Lefevre (1995) The conservation of *Populus nigra* L. and gene flow with cultivated poplars in Europe. Forest Genetics 2: 135-144.

Casiva P, J Vilardi, A Cialdella and B Saidman (2004) Mating system and population structure of *Acacia aroma* and *A. macracantha* (Fabaceae). American Journal of Botany 91: 58-64.

Chakrabarty T and M Gangopadhyay (1996) The genus *Acacia* P. Miller (Leguminosae: Mimosoideae) in India. Journal of Economic Botany 20: 599-633.

Chakraborty R, T R Meagher and P E Smouse (1988) Parentage analysis with genetic markers in natural populations I. The expected proportion of offspring with unambiguous paternity. Genetics 118: 527-536.

Christodloulou S (2003) The impact of *Acacia saligna* invasion on the autochthonous communities of the Akrotiri salt marshes. Honours Thesis. Department of Forestry, University of Central Lancashire. Penrith.

Clarke C, R George, R Bell and T Hatton (2002) Dryland salinity in south-western Australia: its origins, remedies and future research directions. Australian Journal of Soil Research 40: 93-113.

COAG (2000) Our vital resources: A national action plan for salinity and water quality in Australia. Canberra, A.C.T., Council of Australian Governments

Coates D, G Tischler and J A McComb (2006) Genetic variation and the mating system in the rare ghost wattle, *Acacia sciophanes* compared with its common sister species *Acacia anfractuosa* (Mimoseaceae). Conservation Genetics 7: 931-944.

Cogger H (1991) Australia's Reptile Biodiversity. Biolinks 6-7.

Craven L, B J Lepschi, L Broadhurst and M Byrne (2004) Taxonomic revision of the broombush complex in Western Australia (Myrtaceae, *Melaleuca uncinata* s.l.). Australian Systematic Botany 17: 255-271.

Crompton H (1992) *Acacia saligna* - for dryland fodder and soil stabilisation. NFT Highlights. Waimanalo, Hawaii, Nitrogen Fixing Tree Association.

Crowther M, P Spencer, D Alpers and C Dickman (2002) Taxonomic status of the mardo, *Antechinus flavipes leucogaster* (Marsuplialia: Daysyuridae): a morphological, molecular, reproductive and bioclimatic approach. Australian Journal of Zoology 50: 627-647.

Dana E, M Cerrillo, M Sanz-Elorza, E Sobrino and J Mota (2001) Contribución al conocimiento de las xenófitas en España: catálogo provisional de la flora alóctona de Almería. Acata Botanica Malacitana 26: 264-276.

DEST. (1996) The National Strategy for the Conservation of Australia's Biological Diversity. Retrieved from: http://www.environment.gov.au/biodiversity/publications/strategy/index.html.

Devlin B and N C Ellstrand (1990) The development and application of refined methods for estimating gene flow from angiosperm paternity analysis. Evolution 44: 248-259.

Devlin B, K Roeder and N C Ellstrand (1988) Fractional paternity assignment: theoretical development and comparison to other methods. Theoretical and Applied Genetics 76: 369-380.

Dick C (2001) Genetic rescue of remnant tropical trees by an alien pollinator. Proceedings of the Royal Society of London B Biological Sciences 268: 2391-2396.

Dick C, G Etchelecu and F Austerlitz (2003) Pollen dispersal of tropical trees (*Dinizia excelsa*: Fabaceae) by native insects and African honeybees in pristine and fragmented Amazonian rainforest. Molecular Ecology 12: 753-764.

Doran J and J Turnbull (1997) Australian trees and shrubs: species for land rehabilitation and farm planting in the tropics. Canberra, Australia, The Australian Centre for International Agricultural Research.

Dow B and M Ashley (1998) High levels of gene flow in Bur Oak revealed by paternity analysis using microsatellites. Heredity 89: 62-70.

Doyle J and J Doyle (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Duminil J, H Caron, I Scotti, C Saint-Omer and J Petit (2006) Blind population genetics survey of tropical rainforest trees. Molecular Ecology 15: 3505-3513.

Edmands S (2007) Between a rock and a hard place: evaluating the relative risks of inbreeding and outbreeding for conservation and management. Molecular Ecology 16: 463-475.

Edmands S and C Timmerman (2003) Modelling factors affecting the severity of outbreeding depression. Conservation Biology 17: 883-892.

El-Kassaby Y (2000) Effect of forest tree domestication on gene pools. In: Forest conservation genetics: principles and practice. Eds A Young, D Boshier and T Boyle. Collingwood, Victoria, CSIRO: 197-213.

Eldridge M and R Close (1992) Taxonomy of rock wallabies, *Petrogale* (Marsuplialia, Macropodidae). 1. A revision of the Eastern *Petrogale* with the description of 3 new species. Australian Journal of Zoology 40: 605-625.

Elliott C P and M Byrne (2004) Phylogenetics and the conservation of rare taxa in the *Eucalyptus angustissima* complex in Western Australia. Conservation Genetics 5: 39-47.

Ellstrand N (1992) Gene flow by pollen: implications for plant conservation genetics. Oikos 63: 77-86.

Ellstrand N, H Prentice and J Hancock (1999a) Gene flow and introgression from domesticated plants into their wild relatives. Annual Review of Ecology and Systematics 30: 539-563.

Ellstrand N C and Ellam, D (1993) Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics 24: 217-242.

Ellstrand N C, H C Prentice and J F Hancock (1999b) Gene flow and introgression from domesticated plants into their wild relatives. Annual Review of Ecology and Systematics 30: 539-563.

Engels W (1993) Contributing software to the Internet: the *Amplify* program. Trends in Biochemical Sciences 18: 448-450.

Evanno G, S Regnaut and J Goudet (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611-20.

Excoffier L, P Smouse and J Quattro (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

Falush D, M Stephens and J Pritchard (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164: 1567-1587.

Farrington P, R Salama, G Watson and G Bartle (1992) Water use of agricultural and native plants in a Western Australian wheatbelt catchment. Agricultural Water Management 22: 357-367.

Fenster C and M Dudash (1994) Genetic considerations for plant population restoration and conservation. In: Restoration of endangered species: conceptual issues, planning, and implementation. Eds M Bowles and C Whelan. Cambridge, UK, Cambridge University Press: 34-62.

Floyd A G (1987) Status of rainforest in northern New South Wales. In: The Rainforest Legacy, Australian National Rainforests Study. Canberra, Australian Government Publishing Service. 1: 95-118.

Fox J E D (1995) A review of the ecological characteristics of *Acacia saligna* (Labill.) H.Wendl. Mulga Research Centre Journal 12: 39-55.

Friedman S and W Adams (1985) Estimation of gene flow into two seed orchards of loblolly pine (*Pinus taeda* L.). Theoretical and Applied Genetics 69: 609-615.

Garg L, N Bhandari, V Rani and S Bhojwani (1996) Somatic embryogenesis and regeneration of triploid plants in endosperm cultures of *Acacia nilotica*. Plant Cell Reports 15: 855-858.

George N (2005) Koojong (*Acacia saligna*), a species with potential as a perennial forage for dryland salinity management - genetic variation, feed quality and reproductive biology. PhD Thesis. School of Plant Biology, The University of Western Australia. Perth.

George N, M Byrne, B Maslin and G Yan (2006) Genetic differentiation among morphological variants of *Acacia saligna* (Mimosaceae). Tree Genetics and Genomes 2: 109-119.

Georgiades C (1994) The adventive flora of Cyprus, taxonomic, floristic, phytogeographic, ecophysiological study. PhD Thesis. Athens University. Athens.

Gerber S, P Chabrier and A Kremer (2003) FAMOZ: a software for parentage analysis using dominant, codominant and uniparentally inherited markers. Molecular Ecology Notes 3: 479-481.

Ghassemi F, A Jakeman and H Nix (1995) Salinisation of land and water resources, human causes, extent, management and case studies. Sydney, University of New South Wales Press.

Glanznig A (1995) Native vegetation clearance, habitat loss and biodiversity decline: An overview of recent native vegetation clearance in Australia and its implications for biodiversity. Biodiversity Series. Canberra, Department of Environment, Sport and Territories.

Glenn T C and N A Schable (2005) Isolating microsatellite DNA loci. Methods in Enzymology 395: 202-222.

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices. 2.9.3.2 Available at: http://www2.unil.ch/popgen/softwares/fstat.htm.

Hadjikyriakou G and E Hadjisterkotis (2002) The adventive plants of Cyprus with new records of invasive species. Zeitschrift für Jagdwissenschaft 48: 59-71.

Hails R S (2000) Genetically modified plants - the debate continues. Trends in Ecology & Evolution 15: 14-18.

Hamrick J and D Murawski (1990) The breeding structure of tropical tree populations. Plant Species Biology 5: 157-165.

Harwood C, H Thinh, T Quang, P Butcher and E Williams (2004) The effect of inbreeding on early growth of *Acacia mangium* in Vietnam. Silvae Genetica 53: 65-69.

Hatton T and J Ruprecht (2001) Watching the rivers flow. Wheatbelt Valleys Conference, Perth, Waters and Rivers Commission.

Hatton T J, J Ruprecht and R J George (2003) Preclearing hydrology of the Western Australia wheatbelt: Target for the future? Plant and Soil 257: 341-356.

Henderson L (2001) Alien weeds and invasive plants. A complete guide to declared weeds and invaders in South Africa. In: Plant Protection Research Institute Handbook No. 12., Department of Agriculture and Department of Water Affairs and Forestry: South Africa.

Herbert P, A Cywinska, S Ball and J deWaard (2004) Biological identification through DNA barcodes. Proceedings of the Royal Society of London B Biological Sciences 270: 313-321.

Hobbs T, M Bennell, D Huxtable, J Bartle, C Neumann, N George and W O'Sullivan (2006) FloraSearch Species Profiles – Low rainfall farm forestry options for southern Australia. RIRDC Report. Adelaide, S.A., Rural Industries Research and Development Corporation.

Hufford K M and S J Mazer (2003) Plant ecotypes: genetic differentiation in the age of ecological restoration. Trends in Ecology & Evolution 18: 147-155.

Huxel G (1999) Rapid displacement of native species by invasive species: effects of hybridisation. Biological Conservation 89: 143-152.

Huxtable D, J Bartle, G Olsen and W O'Sullivan. (2006) Western Australian field trials involving *Acacia saligna*. Retrieved from: http://www.worldwidewattle.com/infogallery/utilisation/saligna.php.

James T, S Vege, P Aldrich and J Hamrick (1998) Mating system of three tropical dry forest tree species. Biotropica 30: 587-594.

Jones M, M Shepard, R Henry and A Delves (2007) Pollen flow in *Eucalyptus grandis* determined by paternity analysis using microsatellite markers. Tree Genetics and Genomes doi 10.1007/s11295-007-0086-0.

Kang K, D Lindgren and T Mullion (2001) Prediction of genetic gain and gene diversity in seed orchard crops under alternative management strategies. Theoretical and Applied Genetics 103: 1099-1107.

Kaufman S, P Smouse and E Alvarez-Buylla (1998) Pollen-mediated gene flow and differential male reproductive success in a tropical pioneer tree, *Cecropia obtusifolia* Bertol. (Moraceae): a paternity analysis. Heredity 81: 164-173.

Keighery G (2000) Wheatbelt wonders under threat. Landscope 16: 37-42.

Kenrick J (2003) Review of pollen-pistil interactions and their relevance to the reproductive biology of *Acacia*. Australian Systematic Botany 16: 119-130.

Kenrick J and B Knox (1982) Function of the polyad in reproduction of *Acacia*. Annals of Botany (London) 50: 721-727.

Kenrick J and B Knox (1989a) Pollen-pistil interactions in Leguminosae (Mimosoideae). Advances in Legume Biology: Proceedings of the Second International Legume Conference, St Louis, Missouri.

Kenrick J and B Knox (1989b) Quantitative analysis of self incompatibility in trees of seven species of *Acacia*. Journal of Heredity 80: 240-245.

Kondrashov A (1992) The third phase of Wright's shifting-balance: a simple analysis of the extreme case. Evolution 46: 1972-1975.

Kodela P, G (2002) Acacia. In: Flora of New South Wales. Revised edition, Volume 2. Ed. G J Harden. Sydney, NSW, University of New South Wales. 381-476.

Konuma A, Y Tsumura, C Lee and S Lees (2000) Estimation of gene flow in the tropical-rainforest tree *Neobalanocarpus heimii* (Dipterocarpaceae), inferred from paternity analysis. Molecular Ecology 9: 1843-1852.

Kwak M, O Velterop and J VanAndel (1998) Pollen and gene flow in fragmented habitats. Applied Vegetation Science 1: 37-54.

Lambert M and J Turner (2000) Commercial forest plantations on saline lands. Collingwood, Vic, CSIRO Publishing.

Lande R (1988) Genetics and demography in biological conservation. Science 241: 1455-1460.

Latouche-Halle C, A Ramboer, E Bandou, H Caron and A Kremer (2004) Long-distance pollen flow and tolerance to selfing in a neotropical tree species. Molecular Ecology 13: 1055-1064.

Lefroy E and R Stirzaker (1999) Agroforestry for water management in the cropping zone of southern Australia. Agroforestry Systems 45: 277-302.

Lemmetyinen J and T Sopanen (2004) Modification of flowering in forest trees. In: Molecular Genetics and Breeding of forest trees. Eds S Kumar and M Fladung. New York, Food Products Press.

Levin D and H Kerster (1969) The dependence of bee-mediated pollen and gene dispersal upon plant density in *Phlox*. Evolution 23: 560-571.

Levin D and H Kerster (1974) Gene flow in seed plants. Evolutionary Biology 7: 139-220.

Lewis P O and D Zaykin (2001) Genetic Data Analysis: Computer program for the analysis of allelic data. 1.0 (d16c) Available at: http://lewis.eeb.uconn.edu/lewishome/software.html.

Lowe A J, D Boshier, M Ward, C F E Bacles and C Navarro (2005) Genetic resource impacts of habitat loss and degradation: reconciling empirical evidence and predicted theory for neotropical trees. Heredity 95: 255-273.

Lynch M (1991) The genetic interpretation of inbreeding depression and outbreeding depression. Evolution 43: 622-629.

Mandal A and R Ennos (1995) Mating system analysis in a natural population of *Acacia nilotica* subspecies *kraussiana*. Forest Ecology and Management 79: 235-240.

Manel S, P Berthier and G Luikart (2002) Detecting wildlife poaching: identifying the origin of individuals with bayesian assignment test and multilocus genotypes. Conservation Biology 16: 650-659.

Manel S, O E Gaggiotti and R S Waples (2005) Assignment methods: matching biological questions with appropriate techniques. Trends Ecol Evol 20: 136-42.

Marshall T, J Slate, L Kruuk and J Pemberton (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7: 639-655.

Maslin B (1974) Studies in the genus Acacia - 3 – The taxonomy of *A. saligna* (Labill.) H. Wendl. Nuytsia 1: 332-340.

Maslin B (2001) *Acacia saligna*. In: Flora of Australia, Mimosaceae, *Acacia* Part 1. Melbourne, Vic, CSIRO Publishing. 11A: 416-418.

Maslin B and M McDonald (2004) AcaciaSearch: Evaluation of *Acacia* as a woody crop option for southern Australia. Canberra, Rural Industries and Research Development Corporation.

Maslin B, M McDonald and W O'Sullivan. (2006) *Acacia saligna* (Coojong). World wide wattle Retrieved from: http://www.worldwidewattle.com/infogallery/utilisation/saligna.php.

McCartney H, S Foster, B Fraaije and E Warde (2003) Molecular diagnostics for fungal plant pathogens. Pesticide Management Science 59: 129-142.

McDonald M, P Butcher, J Bell and C Nguyen (2003) Clinal variation and genetic divergence in *Acacia tumida* (Mimosaceae). Australian Systematic Botany 16: 57-67.

McDonald M and B Maslin (2000) Taxonomic revision of the salwoods: *Acacia aulacocarpa* Cunn. Ex Benth., and its allies (Leguminosae: Mimosaoideae: section Juliflorae). Australian Systematic Botany 13: 21-78.

McDonald M and B Maslin (in prep) Infraspecific variation in *Acacia saligna* (Labill.) H. Wendl. (*Leguminosae: Mimosoideae*). Australian Systematic Botany.

McFarlane D and R George (1992) Factors affecting dryland salinity in two wheatbelt catchments in Western Australia. Australian Journal of Soil Research 30: 85-100.

McGranahan M, J Bell, G Moran and M Slee (1997) High genetic divergence between geographic regions in the highly outcrossing species *Acacia aulacocarpa* (Cunn. ex Benth.). Forest Genetics 4: 1-13.

Meagher T R (1986) Analysis of paternity within a natural population of *Chamaelirium luteum*. I. Identification of most-likely male parents. American Naturalist 128: 199-215.

Mejnartowicz L (1996) Cisovka - the relic population of *Abies alba* and its relationship to man-made Silver-Fir stands in Bialowieza primeval forest. Acta Societatis Botanicorum Poloniae 65: 319-328.

Michaelides E D (1979) Mini-monograph on, *Acacia cyanophylla* Technical Consultation on Fast-Growing Plantation Broadleaved Trees for Mediterranean and Temperate Zones. Lisbon, Portugal, FAO, Rome.

Midgley S J and J W Turnbull (2003) Domestication and use of Australian acacias: case studies of five important species. Australian Systematic Botany 16: 89-102.

Millar M A and M Byrne (2007) Characterisation of polymorphic microsatellite DNA markers for *Acacia saligna* (Labill.) H.L.Wendl. (Mimosaceae). Molecular Ecology Notes 7: 1372-1374.

Millar M A, M Byrne, I Nuberg and M Sedgley (2008a) High outcrossing and random pollen dispersal in a planted stand of *Acacia saligna* subsp. *saligna* revealed by paternity analysis using microsatellites. Tree Genetics and Genomes. 4: 367-377.

Millar M A, M Byrne, I Nuberg and M Sedgley (2008b) Long distance inter-subspecific gene flow detected by paternity analysis in remnant *Acacia saligna* (Mimosaceae). Heredity. in prep.

Millar M A, M Byrne, I Nuberg and M Sedgley (2008c) A rapid PCR based diagnostic test for the identification of subspecies of *Acacia saligna*. Tree Genetics and Genomes in press. doi 10.1007/s1 1295-008-0138-0

Miller J, B Maslin, R Andrew and R Bayer (2001) Towards an understanding of variation within the Mulga complex (*Acacia aneura* and relatives) using nuclear DNA

techniques. The conservation and utilisation potential of Australian dryland Acacias: Dalwallinu, Western Australia.

Moran G, O Muona and J Bell (1989a) *Acacia mangium*: a tropical forest tree of the coastal lowlands with low genetic diversity. Evolution 43: 251-235.

Moran G, O Muona and J Bell (1989b) Breeding systems and genetic diversity in *Acacia auriculiformis* and *A. crassicarpa*. Biotropica 21: 250-256.

Muona O, G Moran and J Bell (1991) Hierarchical patterns of correlated mating in *Acacia melanoxylon*. Genetics 127: 619-626.

Murawski D and J Hamrick (1991) The effect of the density of flowering individuals on the mating systems of nine tropical tree species. Heredity 67: 167-174.

Muyt A (2001) Bush Invaders of South-East Australia. Meredith, Victoria, RJ Richardson & FJ Richardson.

NAS (1980) Firewood crops. Shrub and tree species for energy production. Washington, D. C., National Academy of Sciences.

Nason J and J Hamrick (1997) Reproductive and genetic consequences of forest fragmentation: two case studies of neotropical canopy trees. Journal of Heredity 88: 264-276.

Nei M (1987) Molecular Evolutionary Genetics. New York, Columbia University Press.

Ng C, S Koh, S Lee, K Ng, A Mark, M Norwati and R Wickneswari (2005) Isolation of 15 polymorphic microsatellite loci in *Acacia* hybrid (*Acacia mangium* x *Acacia auriculiformis*). Molecular Ecology Notes 5: 572-575.

Nielsen R, D Mattila, P Clapham and P Palsball (2001) Statistical approaches to paternity analysis in natural populations and applications to the North Atlantic Humpback Whale. Genetics 157: 1673-1682.

NLWRA (2001) Australian dryland salinity assessment 2000. Extent, impacts, processes, monitoring and management options. Canberra, National Land and Water Resources Audit, Commonwealth of Australia.

Nulsen R, K Bligh, I Baxter, E Solin and D Imrie (1986) The fate of rainfall in a mallee and heath vegetated catchment in southern Western Australia. Agricultural Water Management 4: 173-186.

Nybom H (2004) Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143-1155.

Olsen G, D Cooper, J Carslake, D Huxtable and J Bartle (2004) Developing multipurpose species for large scale revegetation, Search Project Final Report. National Heritage Trust Project 973849. Perth, Western Australian Department of Conservation and Land Management.

Paetkau D, W Calvert, I Stirling and C Strobeck (1995) Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology 4: 347-354.

Paetkau D, R Slade, M Burden and A Estoup (2004) Direct, real-time estimation of migration rate using assignment methods: a simulation-based exploration of accuracy and power. Molecular Ecology 13: 55-65.

Pannel D, D McFarlane and R Ferdowsian (2001) Rethinking the externality issue for dryland salinity in Western Australia. Australian Journal of Agricultural and Resource Economics 45: 459-476.

Parker P, A Snow, M Schug, G Booton and P Fuerst (1998) What molecules can tell us about populations: choosing a molecular marker. Ecology 79: 361-382.

Peakall R and P Smouse (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288-295.

Pearse D and K Crandall (2004) Beyond Fst: Analysis of population genetic data for conservation. Conservation Genetics 5: 585-602.

Piry S, A Alapetite, J Cornuet, D Paetkau, L Baudouin and A Estoup (2004) GENECLASS2: A software for genetic assignment and first generation migrant detection. Journal of Heredity 95: 536-539.

Playford J, J Bell and G Moran (1993) A major disjunction in genetic diversity over the geographic range of *Acacia melanoxylon* R.Br. Australian Journal of Botany 41: 355-368.

Potts B, R Barbour and A Hingston (2001) Genetic pollution from farm forestry using eucalypt species and hybrids. A report for the RIRDC/L&WA/FWPRDC Joint Venture Agroforestry Program. Barton, A.C.T., Rural Industries Research and Development Corporation.

Potts B M, R C Barbour, A B Hingston and R E Vaillancourt (2003) Turner Review No. 6 Genetic pollution of native eucalypt gene pools - identifying the risks. Australian Journal of Botany 51: 1-25.

Pritchard J, M Stephens and P Donnely (2000) Inference of population structure using mulitlocus genotype data. Genetics 155: 945-959.

Rannala B and J Mountain (1997) Detecting immigration by using multilocus genotypes. Proceedings National Academy of Science U.S.A. 94: 9197-9201.

Raybould A F (1999) Transgenes and agriculture - going with the flow. Trends in Plant Science 4: 247-248.

Raybould A F and A J Gray (1994) Will hybrids of genetically modified crops invade natural communities. Trends in Ecology & Evolution 9: 85-89.

Raymond M and F Rousset (1995) GENEPOP (version 3.4): Population genetics software for exact test and ecumenicism. Journal of Heredity 86: 248-249.

Rhymer J M and D Simberloff (1996) Extinction by hybridisation and introgression. Annual Review of Ecology and Systematics 27: 83-109.

Rieseberg L and D Gerber (1995) Hybridisation in the Catalina Island Mountain Mahogany (*Cercocarpus traskiae*): RAPD evidence. Conservation Biology 9: 199-203.

Ritland K (1989) Correlated mating in the partial selfer, *Mimulus guttatus*. Evolution 43: 848-859.

Ritland K (2002) Extensions of models for the estimation of mating systems using *n* independent loci. Heredity 88: 221-228.

Rozen S and H J Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology. Eds S Krawetz and S Misener. Totowa, NJ, Humana Press: 365-386.

Sanz-Elorza M, E Dana and E Sobrino (2001) Aproximación al listado de plantas alóctonas invasoras reales y potenciales en España. Lazzaroa 22: 121-131.

SAS Institute Inc (2006) Base SAS, Procedures Guide, 2nd edition. 9.1.3 Available at: http://www.sas.com/technologies/bi/appdev/base/.

Sato T, Y Isagi, H Sakio, K Osumi and S Goto (2006) Effect of gene flow on spatial genetic structure in the riparian canopy tree *Cercidiphyllum japonicum* revealed by microsatellite analysis. Heredity 96: 79-84.

Scheltema M (1992) Direct Seeding of Trees and Shrubs. Perth, W.A., Greening Western Australia.

Schlötterer C and D Tautz (1992) Slippage synthesis of simple sequence DNA. Nucleic Acids Research 20: 211-215.

Schofield N, I Loh, P Scott, J Bartle, P Ritson, R Bell, H Borg, B Anson and R Moore (1989) Vegetation strategies to reduce stream salinities of water resource catchments in south-west Western Australia. Report to the Steering Committee for Research on Land Use and Water Supply. Perth, Water Authority of Western Australia.

Searle S, J Bell and G Moran (2000) Genetic diversity in natural populations of *Acacia mearnsii*. Australian Journal of Botany 48: 279-286.

Sedgley M, J Harbard, R Smith, R Wickneswari and A R Griffin (1992) Reproductive biology and interspecific hybridisation of *Acacia mangium* and *A. auriculiformis* (Leguminosae: Mimosoideae). Australian Journal of Botany 40: 37-48.

Selkoe K A and R J Toonen (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters 9: 615-29.

Shepard R, R Richardson and F Richardson (2001) Plants of Importance to Australia: A Checklist. Meredith, Victoria, Australia, R.G. and F.J. Richardson.

Short R and C McConnell (2000) Extent and Impacts of Dryland salinity in Western Australia. Canberra, A.C.T.

Simmons (1987) Growing Acacias. Kenthurst, N.S.W., Kangaroo Press.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science 236: 787-92.

Smith R and K Sytsma (1990) Evolution of *Populus nigra* (sect. *Aiegeiros*): introgressive hybridisation and the chloroplast contribution of *Populus alba* (sect. *Populus*). American Journal of Botany 77: 1176-1187.

Smouse P, R Dyer, R Westfall and V Sork (2001) Two generation analysis of pollen flow across a landscape. I. Male gamete heterogeneity among females. Evolution 55: 260-271.

Spies B and P Woodgate (2003) Review of salinity mapping methods in the Australian context. Draft Technical Report. Canberra, A.C.T., Natural Resource Management Standing Committee.

SPSS (2001) SPSS for Windows Student Version. Rel 11.0 Available at: http://www.spss.com/spss16/.

Stacy E, J Hamrick, J Nason, S Hubbell, R Foster and R Condit (1996) Pollen dispersal in low-density populations of three neotropical tree species. American Naturalist 148: 275-298.

Stirzaker R, R Vertessy and A Sarre (2002) The purpose of this book. In: Trees, water and salt. An Australian guide to using trees for healthy catchments and productive farms. Eds R Stirzaker, R Vertessy and A Sarre. Barton, A.C.T., Rural Industries Research and Develpoment Corporation.

Stone G N, N E Raine, Prescott and P G Willmer (2003) Pollination ecology of acacias (Fabaceae, Mimosoideae). Australian Systematic Botany 16: 103-118.

Taylor R and G Hoxley (2003) Dryland Salinity in Western Australia: Managing a changing water cycle. Water Science and Technology 47: 201-207.

Templeton A (1986) Coadaption and outbreeding depression. In: Conservation Biology: The Science of Scarcity and Diversity. Ed. M Soule. Sunderland, MA, Sinauer: 105-116.

Tiedje J M, R K Colwell, Y L Grossman, R E Hodson, R e Lenski, R N Mack and P J Regal (1989) The planned introduction of genetically engineered organisms: ecological considerations and recommendations. Ecology 70: 298-315.

Valbuena-Carabana M, S Gonzales-Martinez, V L Sork, C Collada, A Soto and P Goicoechea (2005) Gene flow and hybridisation in a mixed oak forest (*Quercus pyrenaica* Willd. and *Quercus petraea* (Matts.) Liebl.) in central Spain. Heredity 95: 457-465.

Weir B and C Cockerham (1984) Estimating F-statistics for the analysis of population structure. Evolution 38: 1358-1370.

Wheeler N and K Jech (1992) The use of electrophoretic markers in seed orchard research. New Forests 6: 311-328.

White G, D Boshier and W Powell (2002) Increased pollen flow counteracts fragmentation in a tropical dry forest: an example from *Swietenia humilis* Zuccarini. Proceedings of the National Academy of Sciences USA 99: 2038-2042.

Wickneswari R and M Norwati (1993) Genetic diversity of natural populations of *Acacia auriculiformis*. Australian Journal of Botany 41: 65-77.

Williams J and D A Saunders. (2003) Land use and natural ecosystems: A revolution in land use is the key to a sustainable landscape. In Search of Sustainability Retrieved from: http://www.isoconference.org.au.

Williamson D (1998) Land Degradation Processes and Water Quality Effects: Waterlogging and Salinisation. In: Farming Action - Catchment Reaction: the effect of dryland farming on the natural environment. Eds J Williams, R Hook and H Gascoigne. Collingwood, Victoria, Australia, CSIRO Publishing: 162-187.

Wolf D E, N Takebayashi and L H Rieseberg (2001) Predicting the risk of extinction through hybridisation. Conservation Biology 15: 1039-1053.

Working Groups on Biodiversity (1992) Scientific Aspects of Major Environmental Issues: Biodiversity. Canberra, Paper prepared for consideration by the Prime Minister's Science Council at its sixth meeting, 18 May 1992. Papers produced by the Office of the Chief Scientist, Department of Prime Minister and Cabinet.

Yeh F, R Yang, B Boyle, Z Ye and J Mao (1997) POPGENE: The User Friendly Shareware for Population Genetic Analysis. In: Molecular Biology and Biotechnology Centre. Alberta, Canada, University of Alberta.

Yelenick S G, W D Stock and D M Richardson (2004) Ecosystem level impacts of invasive *Acacia saligna* in the south African Fynbos. Restoration Ecology 12: 44-51.

Young A, T Boyle and A Brown (1996) The population genetic consequences of habitat fragmentation for plants. Trends in Ecology & Evolution 11: 413-418.

Zane L, L Bargelloni and T Patarnello (2002) Strategies for microsatellite isolation: a review. Molecular Ecology 11: 1-16.

Zhang D and G Hewitt (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Molecular Ecology 12: 563-584.

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