Pollen contamination in *Acacia saligna*: assessing the risks for sustainable agroforestry

M. A. Millar[1, 2, 3] & M. Byrne[2, 3]
[1]The University of Adelaide, Australia
[2]CRC for Plant Based Management of Dryland Salinity, Australia
[3]Department of Environment and Conservation, Australia

Abstract

Species developed for sustainable agroforestry may pose risks to remnant populations of closely related species via genetic contamination. Genetic contamination and the production of hybrid progeny may threaten the health and long-term viability of remnant populations. *Acacia saligna* is a native Western Australian species complex selected for further development for agroforestry in the agricultural areas of southern Australia. *A. saligna* shows great morphological, ecological, biological and genetic variation, and will be reclassified into a number of subspecies. This research aimed to develop genetic markers and use them to assess the levels and distances of gene flow via pollen dispersal between two of the proposed subspecies of *Acacia saligna*. Pollen dispersal from the abundantly flowering subsp. *saligna* into the poorer flowering subsp. *lindleyi* was high (32%). At the same site, pollen dispersal from subsp. *lindleyi* into subsp. *saligna* was much lower (14%). Most genetic contamination from subsp. *saligna* into subsp. *lindleyi* occurred at short distances (<300 m) although inter-subspecific pollen dispersal was detected over large distances of around 1600 m. The comparative levels of inter-subspecific pollen dispersal are influenced by the relative floral fecundity of the different variants. The results indicate that small remnant populations of subsp. *lindleyi* will be exposed to high levels of hybridisation when subsp. *saligna* is planted nearby for agroforestry. The effects of inter-subspecific hybridisation in *A. saligna* are unknown and until further investigated it is recommended as a management guideline that isolation distances of 1600 m or more be imposed between agroforestry plantings and native populations.
Keywords: agroforestry, pollen dispersal, species complex, inter-subspecific hybridisation, genetic contamination, microsatellites, paternity analysis, Acacia saligna, risk assessment, isolation distances.

1 Introduction

Species developed for sustainable agroforestry may pose risks to remnant populations of closely related species when introduced nearby. These risks include direct invasiveness into natural ecosystems, supporting increased incidences of pests, disease and feral animals, and the possibility of genetic contamination in remnant natural populations [1]. Genetic contamination, or the movement of foreign genes from non-local populations into native populations, may occur in tree species via pollen dispersal [2] and may arise in natural populations when populations of a similar species or genetically divergent populations of the same species are planted nearby. If the floral phenologies of divergent populations overlap and sufficient interfertility exist between them, gene flow via pollen transfer between the populations results in intra specific, inter-subspecific or interprovenance hybridisation and the production of hybrid progeny. The likelihood and possible impact of this scenario is receiving increasing attention, due to the threat of gene flow and hybridisation between natural populations and introduced transgenic crops [3-7].

Divergent populations may be intentionally introduced throughout the range of natural populations for agriculture or agroforestry, or through revegetation programs, and unintentionally through the spread of invasive species. In the case of agroforestry, genetic divergence between natural and introduced populations may arise through the use of non-local subspecies or non-local provenance germplasm, or selection pressure applied in breeding and domestication programs [1]. If agroforestry populations (pollen source) are large in comparison to natural remnant populations (pollen sink), levels of genetic contamination and the production of hybrid progeny may be significant.

Genetic contamination in natural populations results in one of two outcomes for hybrid progeny: heterosis or outbreeding depression, and the impacts of either may threaten the health and long-term viability of natural populations [2, 8]. Heterosis, or hybrid vigour, occurs when hybrid fitness is enhanced relative to the parental populations, and may result in the establishment of large hybrid populations between agroforestry and remnant populations. If hybrid fitness is maintained over the generations, the natural population may be at risk from direct competition, hybrid swamping and the impacts of small population processes such as a loss of genetic diversity. Continued hybridisation may eventually result in total genetic assimilation of the typically smaller, native population via introgression. This occurs with the repeated backcrossing of hybrids to one or both of the parental populations.

The other possible outcome for hybrid progeny is outbreeding depression. This occurs when hybrid fitness is reduced relative to the parental population. In this case, the reproductive output of a native population may again be lowered to levels that threaten the population viability [9]. Pollen swamping may also be detrimental to the viability of natural populations regardless of whether hybrid progeny are produced. Seed production and recruitment within the natural population may be significantly reduced, especially in small populations [10, 11].

In Australia, the majority of the once extensive woody vegetation has been cleared for agricultural production and there is an urgent need to revegetate with native perennials. Revegetation is required in order to halt the degradation of biological integrity, restore the hydrological balance and mitigate increasing levels of salinisation in the land and waterways. A number of native Australian species are being developed for use in revegetating the agricultural areas of southern Australia via agroforestry; however, there is some risk to remnant populations via genetic contamination with large-scale agroforestry.   The potential for genetic contamination and hybridisation between non-local populations of native species needs to be considered to ensure that revegetation systems are environmentally sustainable and pose no further risks to biodiversity. The potential for genetic contamination from large-scale agroforestry crops must be evaluated within a risk management framework that requires comprehensive risk assessment [8, 12].

Acacia saligna is a native Western Australian species complex selected for further development for agroforestry, as a short rotation phase or coppice crop, in the low rainfall areas of southern Australia [13, 14]. *A. saligna* has been recognised as a species complex that shows great morphological, ecological, biological and genetic variation. The species complex has been partitioned into a number of subspecies to be formally taxonomically described. Little is known about the mating system, patterns of gene flow via pollen dispersal, and levels of genetic contamination that can be expected within and between populations of the different subspecies of *A. saligna*. These parameters can be estimated through paternity analysis using genetic markers such as microsatellites. Microsatellite markers are suitable for paternity analysis studies as they are highly polymorphic, codominant, considered selectively neutral, and can be amplified from small amounts of genomic DNA.

As part of a larger process of assessing the risk posed by large-scale utilisation of *A. saligna* as an agroforestry crop to natural remnant populations of *A. saligna*, we developed and screened microsatellite markers for use in *A. saligna* [15].

The markers were then used for paternity analysis to investigate pollen dispersal patterns and levels of genetic contamination, via inter-subspecific hybridisation, for two of the proposed subspecies of *A. saligna*. The results of this work will be used in developing risk management protocols that ensure the environmentally sustainable use of *A. saligna* for agroforestry throughout southern Australia.

2   Materials and methods

2.1 Study species

Acacia saligna is a leguminous bushy shrub or tree, naturally distributed over southwestern Australia where it experiences a Mediterranean climate. Trees grow to 2-10 m at maturity and live for up to twenty years under favourable conditions [16].   Trees bear hermaphroditic, many flowered, globular

inflorescences and produce flat pods containing small, hard, black seeds [17]. The species complex consists of four main variants to be formally described as subspecies (McDonald MW and Maslin BR ms): illustrated in Figure 1.
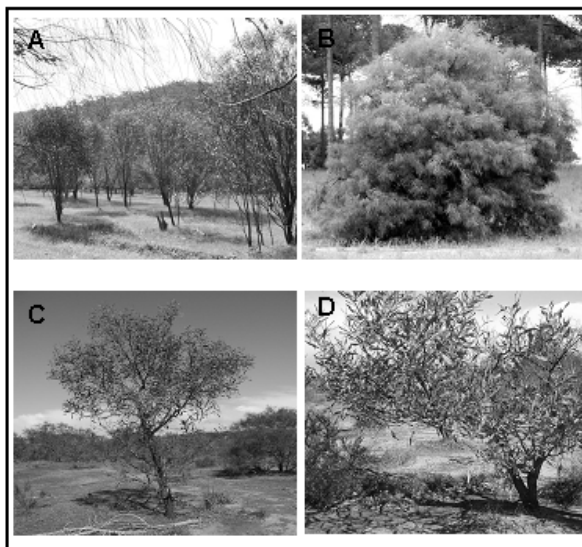

Figure 1:   Morphological variants of *Acacia saligna* showing mature specimens of subspecies (A) *lindleyi* (B) *saligna* (C) *stolonifera* and (D) pruinescens.

Acacia saligna subsp. *saligna* is native to the coastal areas around Perth and on the south coast; it 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 variant 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. It has a spindly form with little biomass and limited terminal flowering. *Acacia saligna* subsp. *stolonifera* has a natural distribution in the forest areas to the south-west of the range of subsp. *lindleyi*. Subsp. *pruinescens* occurs over a restricted area and shows some genetic affinity with subsp. *saligna*.

2.2 Study site and sample collection

The study site consists of a planted stand of 107 trees of *A. saligna* subsp. *saligna* within the natural distribution of subsp. *lindleyi*, trees of which occur as roadside remnants at varying distances from the planted stand (Figure 2). The planted *A. saligna* stand occupies an area of approximately 0.55 hectares at the intersection of two roads. The closest subsp. *lindleyi* trees are approximately 25 m from the edge of the planted stand. This remnant consists of several hundred

individuals, although the majority of these were not reproductively mature at the time of sampling. Trees extend away from the planted stand, along a road verge, in a westerly direction, where they become less abundant, eventually occurring as individuals at a maximum distance of 300 m from the stand. Further remnant subsp. *lindleyi* trees are present along the roadside in a south-easterly direction, where they occur sporadically in two main patches approximately 900 and 1500 m from the stand. Many of the trees in the remnant patches were juvenile and fecundity of the adult trees was highly variable.
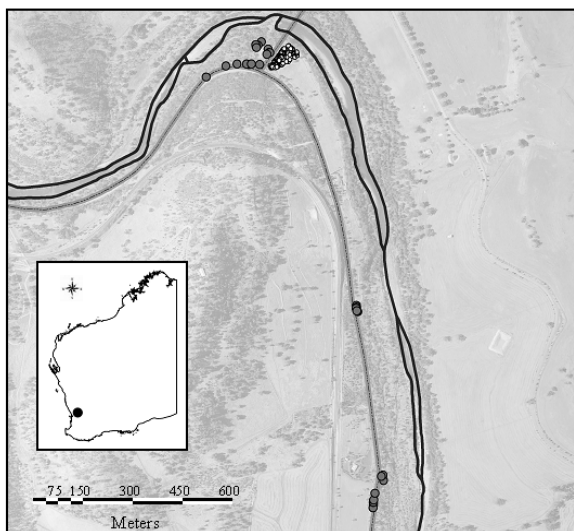

Figure 2:   Map of the study site showing locations of sampled trees of *Acacia saligna*. Larger grey circles represent sampled trees of subsp. *lindleyi*; smaller white circles represent sampled trees of subsp. *saligna* in the planted stand; dark lines represent the Avon River; light grey broken lines represent roadways.

Mature phyllode material was collected from all trees of subsp. *saligna* within the planted stand and from 29 trees of subsp. *lindleyi*. Open pollinated seed was sampled from 10 maternal trees of *A. saligna* subsp. *saligna* chosen at random from within the stand and from each of the selected 29 trees of subsp. *lindleyi*. As seed set was poor in subsp. *lindleyi*, trees with the largest seed crops were chosen for sampling. All trees of subsp. *saligna* and the 29 maternal trees of subsp. *lindleyi* were mapped using a differential global positioning satellite system. Seed from each tree was extracted from dry pods and bulked, sterilised, left to imbibe overnight and planted. No seed germinated for one of the maternal trees of subsp. *saligna* and for two of the trees of subsp. *lindleyi*, so seed crops were analysed for nine and 27 maternal trees respectively. Genomic DNA was extracted from phyllodes of the adult trees using a modified cetyltrimethyl

ammonium bromide (CTAB) extraction method [18], with the addition of 0.1 M sodium sulphite to the extraction and wash buffers [19], and from germinated seedlings using a modified Doyle and Doyle method [20].

Two microsatellite markers developed for *A. saligna* and three microsatellite markers developed for *A. mangium* [21] produced suitable cross amplification in *A. saligna*. Genotypes of all *A. saligna* subsp. *saligna* trees in the planted stand and progeny arrays for nine families consisting of 11-22 seedlings (186 in total), and of 29 *A. saligna* subsp. *lindleyi* trees and progeny arrays for 27 families consisting of 1 –25 seedlings (396 in total), were determined for the five microsatellite loci. 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).

2.3 Data analysis

Paternity assignment was conducted using maximum likelihood methods implemented in CERVUS 2.0 [22]. A 95% criterion was used for assignment, with mismatch at one locus allowed. Since geitonogamous pollination is possible, assignment of paternity to the mother plant was assumed to be due to selfing. The patterns of pollen dispersal within and between trees of subsp. *saligna* and trees of subsp. *lindleyi* were evaluated by determining the distance between mother and pollen source for each of the progeny.

3　Results

Using the five loci, the majority (93.5%) of subsp. *saligna* progeny were assigned a most likely father from either trees of subsp. *saligna* or the sampled subsp. *lindleyi* trees. The majority of subsp. *saligna* progeny (86%) were sired by pollen from within the stand of subsp. *saligna*. Fourteen subsp. s*aligna* progeny (7.5%) were assigned a most likely father from the sampled subsp. *lindleyi* trees. Most likely fathers were undefined for twelve subsp. s*aligna* progeny (6.5%) and these were assumed 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. This inter-subspecific gene flow totalled 14% [15]. Genetic contamination rates varied among maternal trees of subsp. s*aligna* within the stand (0 to 32%). Of the 29 genotyped trees of *A. saligna* subsp. *lindleyi*, 10 (34.5%) were assigned as fathers of one or more progeny of maternal trees in the planted stand of subsp. *A. saligna*. Inter-subspecific pollination events occurred over a range of distances from 5 m to 1566 m with an average pollen dispersal distance of 666 m.

A high percentage of progeny (96.5%) of subsp. *lindleyi* trees were assigned a most likely father from sampled trees of either subsp. *saligna* or subsp. *lindleyi*. Most likely fathers were undefined for fourteen subsp. *lindleyi* progeny (3.5%) and these were assumed sired by unsampled trees of the background population of subsp. *lindleyi*. The rate of pollen contamination in subsp. *lindleyi* trees is the

percentage of subsp. *lindleyi* progeny assigned a most likely father from trees of subsp. *saligna* and totalled 32% (126 progeny) [23].   Inter-subspecific hybridisation in subsp. *lindleyi* was highly variable among maternal trees (0 to 100%) although this was not related to distance from the planted stand. Of the 107 trees of subsp. *saligna*, 45 (42%) were assigned as fathers of one or more progeny in the sampled seed crops of subsp. *lindleyi* trees.   Inter-subspecific pollination distances ranged from 42 m to 1607 m for subsp. *lindleyi* progeny.

4   Conclusions

Inter-subspecific hybridisation from a planted stand of trees of subsp. *saligna* was considerable for remnant roadside populations of subsp. *lindleyi*, and accounted for nearly one-third (32%) of all outcrossed progeny of *lindleyi* trees. At the same site, pollen dispersal from subsp. *lindleyi* into subsp. *saligna* was much lower (14%). These results show that genetic contamination between the subspecies of *A. saligna* will be an issue for the sustainable use of the species as an agroforestry crop throughout its natural range in southern Western Australia. Remnant roadside populations of subsp. *lindleyi* may be at risk of high levels of inter-subspecific hybridisation with introduced populations of subsp. *saligna* throughout the wheatbelt of Western Australia.

The disparity in inter-subspecific hybridisation between the two subspecies of *A. saligna* is likely due to differences in the total amount of pollen produced by each subspecies. Pollen production is influenced by the size of a population and its floral density [24]. Small populations are likely to experience a relatively large proportion of pollen immigration compared to larger populations and pollen immigration will increase in remnant populations as the introduced populations increase in size.   Populations with poor flowering and pollen production are also 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 shows low seed set. In contrast, subsp. *saligna* shows the greatest biomass, floral density, and seed set of all four subspecies [25]. The disparity in floral fecundity between the two subspecies is noticeable at the study site where it would result in trees of subsp. *saligna* producing substantially more pollen in comparison to trees of subsp. *lindleyi*. Pollen production may also be affected by the age composition of populations at the study site. All trees in the planted stand were mature and flowered profusely in 2005, while the remnant populations were comprised of trees of various ages, including many juveniles that produced relatively few flowers and little pollen. This disparity in relative pollen production would lead to a greater contaminant pollen source for subsp. *lindleyi*, at the same time as a greater non-contaminant pollen source for subsp. *saligna*, and have the effect of both increasing inter-subspecific hybridisation in subsp. *lindleyi* and decreasing it in subsp. *saligna*. The disparity in relative pollen production results in greater genetic contamination in subsp. *lindleyi* trees despite the greater number of trees of this subspecies at the study site. 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 genetic contamination can be expected in remnant populations from plantings of subsp. *saligna* once trees reach maturity.

Most genetic contamination from subsp. *saligna* into remnant trees of subsp. *lindleyi* occurred at short distances of less than 300 m although significant levels of inter-subspecific pollen dispersal were detected over large distances of up to 1566 m.   Inter-subspecific genetic contamination in trees of subsp. *saligna* from pollen from subsp. *lindleyi* trees was also detected over long distances of up to 1607 m. The results indicate that while the majority of inter-subspecific pollen contamination from introduced plantings is likely to occur over short distances, substantial amounts of inter-subspecific hybridisation may occur even when introduced populations are located at least 1.5 km away.

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 given taxa, but adverse effects may occur even when levels of inter-subspecific gene flow are low [26].   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.

This study demonstrates that introduced plantings of tree species that maintain extensive levels of long distance gene flow, may pose some risk via pollen dispersal into genetically divergent natural populations. This risk can be reduced through cultural management practices, such as restricting germplasm used for introduced 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 four subspecies are slightly different, but do overlap so the first option may be impossible 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 [27] thus may be harvested before trees are old enough for flowering and pollen production to commence.

The most reliable short-term practice to limit pollen-mediated gene flow in of *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.

Significant levels of inter-subspecific pollen dispersal from subsp. *saligna* into subsp. *lindleyi* was detected over large and isolation distances greater than 1500 m are recommended to prevent significant levels of gene flow into natural populations.

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