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Project Title: Genetic and Ecological Viability of Plant Populations in Remnant Vegetation

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1. PROJECT OBJECTIVES:

The general aim of this research project was to understand and quantify how genetic and demographic processes interact to influence the viability and long-term conservation value of native plant populations in remnant vegetation, and relate this to easily measured landscape and population parameters. This information can then be used to identify and prioritize high viability remnants for *in-situ* conservation and target them as sources of high quality seed for revegetation and restoration. The project also aimed to test conservation genetics theory regarding the genetic deterioration of small fragmented populations that has been developed based on data from rare plants, and to determine how applicable these paradigms are when dealing with common species. This is important since it is the more abundant species that are the critical components of landscapes with regard to maintenance of broader ecosystem function such as hydrology and nutrient cycling, as well as provision of habitat for other native organisms.

Specific research objectives were:

1. Identify and quantify the genetic and demographic factors that affect the viability of plant populations in vegetation remnants. The focus will be on the effects of genetic erosion, inbreeding and pollinator limitation on seed production and seedling fitness. This will involve the integrated use of molecular genetic tools and demographic monitoring to examine four target taxa with varied ecologies.

2. Examine and model the relationships between key genetic and demographic factors affecting viability and remnant vegetation characteristics such as size, disturbance and landscape position.

3. Compare results among four target taxa with varied ecologies to assess how life-history affects the impact of remnant characteristics on population viability.

4. Based on 1-3 generate specific genetic and demographic guidelines for management of remnant populations of the four target taxa and general landscape design principles for major plant *life-history* types that will maximise the probability of population persistence.

2. METHODS

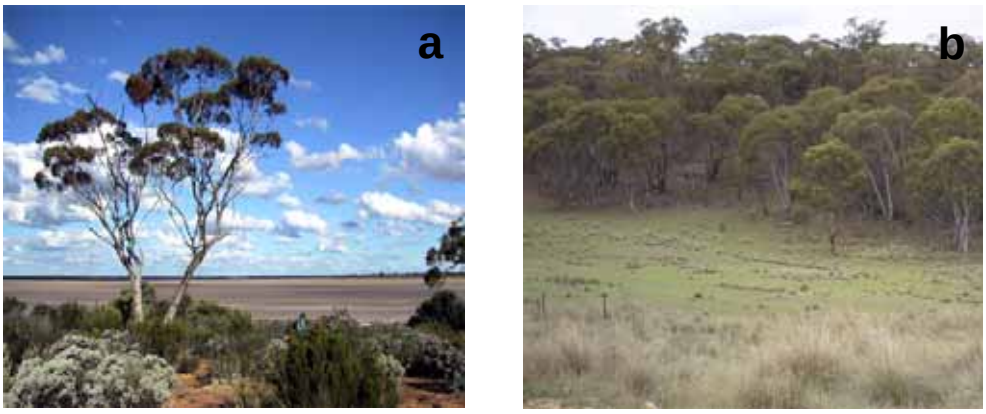
The project was conducted over two field seasons (2002-03 and 2003-04) in two contrasting biomes from New South Wales and Western Australia, using seven *target taxa*. It adopted a multidisciplinary approach that integrated the use of field-based demographic monitoring, experimental growth trials and extensive use of molecular marker analyses to assess population viability focusing on reproduction.

2.1 Biomes

The study was conducted in two contrasting biomes, the temperate grassland-woodland ecosystem in New South Wales and the kwongan shrublands of the Dongolocking region in Western Australia. These ecosystems have been subject to severe habitat loss and disturbance over the last century, and now exhibit a broad range of extent and condition of remaining vegetation. While both represent severely fragmented vegetation communities, the two ecosystems differ in their landscape structure. The kwongan shrubs represent a truly fragmented landscape, with remnant vegetation physically isolated by intervening intensive wheat and grazing land use (Fig. 1a). In contrast, in the east, the grassland-woodland ecosystem represents a more variegated landscape (McIntyre and Hobbs 1999), with intact remnants imbedded in a

less clearly defined matrix of improved native pastures in varying degrees of condition (Fig. 1b). This point of contrast, and its possible effect on the demographic and genetic function of fragmented populations, was a key reason for selecting these two biomes.

Figure 1. *Study Biomes: a) Fragmented kwongan shrubland ecosystem in Western Australia (photograph - Colin Yates) . b) Variegated grassland-woodland ecosystem in New South Wales (photograph – Linda Broadhurst).*



2.2 Target taxa and study populations

Within each biome target taxa were chosen to include, as much as possible, different life forms, pollination and seed dispersal syndromes and longevity, as all of these characteristics are likely to influence both demographic and genetic responses to ecosystem fragmentation. Species were also chosen that were known to occur across a range of fragmentation conditions in terms of remnant size, isolation and disturbance. Given the intensive nature of the combined field monitoring and genetic analysis approach it was initially proposed to study four target taxa , two in each biome. During the course of the study a further three species were added through the use of additional partner agency resources. For these taxa not all elements of the study were conducted on each species. The final target taxa and their ecological characteristics are listed in Table 1 along with the number of populations of each that were used in the study. Species descriptions and distributions are in Appendix 7.1.

TABLE 1 Target taxa, ecological characteristics and study populations

Taxa	Biome	Life form	Pollination	Dispersal	Longevity	Number of populations
Eucalyptus aggregata	SE	Tree	Insect	Wind/gravity	>100y	18
Acacia dealbata	SE	Tree	Insect	Bird/gravity	>20y	16
Acacia acinacea	SE	Shrub	Insect	Bird/gravity	>20y	35
Swainsona sericea	SE	Herb	Insect	Insect/gravity	>5y	10
Eucalyptus wandoo	WA	Tree	Bird/insect	Wind/gravity	>100y	19
Calothamnus quadrifidus	WA	Shrub	Bird/mammal	Gravity	>40y	20
Eremaea pauciflora	WA	Shrub	Insect	Gravity	>40y	19

2.3 Landscape and population parameters

Study populations for each target taxa were chosen from among available remnants to cover the range of population size, isolation and condition observed in each landscape. Population sizes were determined either by direct counts of reproductive individuals, or by estimating plant densities and multiplying by remnant area. Population isolation was estimated differently in the two biomes. In the kwongan shrublands, isolation was measured as the percentage area of native vegetation within a 3km radius of the study population. In the grassland-woodland biome, it was not possible to take this approach owing to the enormous variation in matrix condition among remnants. In this biome, isolation was estimated as the average distance to the five nearest native vegetation remnants. Each remnant was also scored for disturbance which was measured as weed abundance based either on percent exotic cover measured using 1m quadrats or the ratio of exotics to natives across the whole site.

2.4 Genetic variation and inbreeding

Genetic analysis of all populations was undertaken using either co-dominant or dominant molecular markers as indicated in Table 2 (See Appendix 7.4 Scientific Papers for details of methods) to quantify the amount and distribution of genetic variation within and among populations and to determine levels of inbreeding. Key measures of genetic variation estimated were: 1) polymorphism (*P*) the number of loci assayed that exhibit allelic variation, 2) allelic richness (*A*) the average number of alleles per locus and, 3) gene diversity (H_e) which integrates allelic richness and allele frequencies to give an overall measure of genetic diversity. Inbreeding was estimated indirectly using the fixation coefficient (F_{is}), which measures the deficit of observed heterozygosity against that expected assuming random mating. Inbreeding was also measured directly by estimating the proportion of outcrossed seed in the seed cohort (*tm*). When co-dominant markers were available, the effective size of the pollen pool was also determined by calculating the correlation of outcrossed paternity (*rp*).

TABLE 2 Molecular markers used and genetic diversity and inbreeding parameters estimated for each target taxa.

Taxa	Markers	Cohort sampled	*P*	*A*	H_e	F_{is}	*tm**	*rp**
Eucalyptus aggregata	Allozymes	Seed	■	■	■	■	■	■
Acacia dealbata	Allozymes	Seed	■	■	■	■	■	■
Acacia acinacea	AFLPs	Seed	■	■	■		■	■
Swainsona sericea	AFLPs	Seed	■	■	■		■	
Eucalyptus wandoo	RFLPs/SSRs	Adults /seed	■	■	■	■	■	■
Calothamnus quadrifidus	Allozymes	Seed	■	■	■	■	■	■
Eremaea pauciflora	Allozymes	Seed		■	■	■		

■ Parameters estimated for each species.

*estimated on a subset of 5-10 populations sampled across the observed range of population size and condition.

In each population up to 30 individuals were genotyped for estimates of genetic diversity. Up to 20 seedlings were germinated from open-pollinated seed crops from each of up to 15 reproductive individuals and genotyped to estimate outcrossing rate and correlated paternity following (Ritland 1989, 1990 and 2002) (see Appendix 7.4 Scientific Papers for details of methods). Sampling for estimation of genetic parameters was conducted in a single season.

2.5 Landscape-level gene flow

For *Eucalyptus wandoo*, the availability of high-resolution SSR markers allowed some direct analysis of landscape level gene flow. For this species all adults in the five smallest populations (n=2, 5, 9, 40 and 46) were genotyped and genotypes from the seed harvested from reproductive plants in these populations were subject to paternity exclusion analysis. This allowed the proportion of seed sired on trees in a remnant by pollen coming from outside the population to be determined, giving a direct measure of the frequency of interpopulation gene flow and the importance pollen immigration to fecundity.

2.6 Pollination, fecundity and fitness

Pollinator visitation (*C. quadrifidus*), pollination, fertilization (*C. quadrifidus* and *E. wandoo*), flowering, fruit maturation, seed set, germination and seedling fitness under controlled conditions (all target taxa except *E. aggregata* and *Eremaea pauciflora*) were estimated over either one or two reproductive seasons depending on flowering (see Appendix 7.4 Scientific Papers for details of methods) for a subset of 5-15 populations for each species sampled across the observed range of population size and condition (Table 3). For *A. dealbata* seed from both reproductive seasons was pooled into a single growth experiment owing to low seed set.

TABLE 3 Reproductive and growth parameters measured for each target taxa.

Taxa	Seasons	Flowering	Pollinator visits	Pollination	Seed set	Germination	Growth
Acacia dealbata	2	■		■	■	■	■
Acacia acinacea	1					■	■
Swainsona sericea	1	■			■	■	■
Eucalyptus wandoo	2			■	■	■	■
Calothamnus quadrifidus	2		■	■	■	■	■

■ Parameters estimated for each species.

2.7 Estimation of relationships between population and landscape characteristics and genetic and demographic response variables

Univariate and multiple linear regression models were used to explore relationships between population size, isolation and disturbance as independent variables and measures of genetic variation, inbreeding, fecundity and fitness as dependent variables separately for each species (see Appendix 7.4 Scientific Papers for details of methods).

3. RESULTS

This study represents the first large-scale, multi-species, investigation of the genetic and demographic response of common plant species to landscape fragmentation. Approximately 6000 individuals were genotyped for multiple loci across 137 populations to measure variation and inbreeding, combined with assessment of population fecundity and seedling fitness under controlled conditions.

3.1 Genetic variation and inbreeding

Analysis of genetic variation and mating system and their relationship to population and landscape characteristics aimed to determine which genetic parameters, if any, were affected significantly by habitat fragmentation, and to quantify their relationship to population size, isolation and disturbance. Previous work in this area has focused on rare plants e.g. van Treuren, *et al.* (1991); Oostermeijer *et al.* (1994); Raijmann *et al.* (1994); Young *et al.* (1999). In these studies strong positive relationships between population size and genetic diversity and outcrossing rates have been observed suggesting dramatic reductions in the genetic quality and fitness of individuals in small populations. This would severely reduce the conservation value of small vegetation remnants. If this is true for common species, previous studies that have focused on quantifying relationships between species richness and vegetation remnant attributes may well overestimate long-term species persistence within landscapes by confusing current presence with long-term viability.

Key research questions were:

1) Do small, isolated or disturbed populations exhibit reduced genetic variation, in particular heterozygosity?
2) Do small isolated or disturbed populations show an increase in inbreeding that could reduce fitness?
3) Are there tradeoffs between landscape parameters in their effects, for example a tradeoff between isolation and population size, that point to landscape design principles?
4) Can these data be used to determine minimum remnant thresholds to maintain genetically and demographically viable populations?

Results of regression analyses (Table 4 and Appendix 7.2) indicated that:

1) Common species do experience significant genetic erosion as evidenced by loss of *P* and *A* as remnant population size decreases.
2) The genetic response to reduced population size is variable among species, and not easily predicted based on life-history.
3) There is little if any evidence of increased selfing (direct inbreeding) in small populations of any species. However, when estimates of selfing and biparental inbreeding (outcrossed mating among relatives - estimated as *tm-t*s following Ritland (1990)) are combined *E. wandoo* show elevated inbreeding with reduced population size. This is a very important result as inbreeding is the primary mechanism that will negatively effect seedling fitness through expression of inbreeding depression, under either overdominace or increased expression of deleterious recessive alleles.
4) Correlated paternity was also sensitive to population size, with smaller populations of *C. quadrifidus* showing greater correlation among paternal genetic contributions to seed, indicating reduced effective pollen pools. For *A. acinacea* increasing isolation also increased correlated paternity. These are critical as higher *rp* increases relatedness in the next generation, which will exacerbate biparental inbreeding effects in the future.

5) Compared to population size, the other two population/landscape parameters, isolation and disturbance, had only occasional and unpredictable effects on the measured genetic variables.

Table 4 Effect of population size, isolation and disturbance on genetic variation and inbreeding.

Taxa	Pop/landscape parameter	*P*	*A*	H_e	F_{is}	*tm*	*rp*
E. aggregata	Size	++	++	ns	ns	ns	ns
A. dealbata	Size	++	++	ns	ns	--	ns
	Isolation	ns	--	ns	ns	+	ns
	Disturbance	--	ns	ns	ns	ns	ns
A. acinacea	Size	ns	ns	ns		ns	ns
	Isolation	ns	ns	ns		ns	++
	Disturbance	ns	ns	ns		ns	
S. sericea	Size	ns	ns	ns		ns	
	Isolation	--	ns	--		ns	
	Disturbance	ns	ns	ns		ns	
E. wandoo	Size	++	++	ns	ns	ns	ns
	Isolation		ns	ns	ns	+	ns
	Disturbance		ns	ns	ns	ns	ns
C. quadrifidus	Size	++	++	++	ns	ns	--
	Isolation		ns	ns	ns	ns	--
	Disturbance		ns	ns	ns	ns	ns
Eremaea pauciflora	Size		ns	ns	ns		

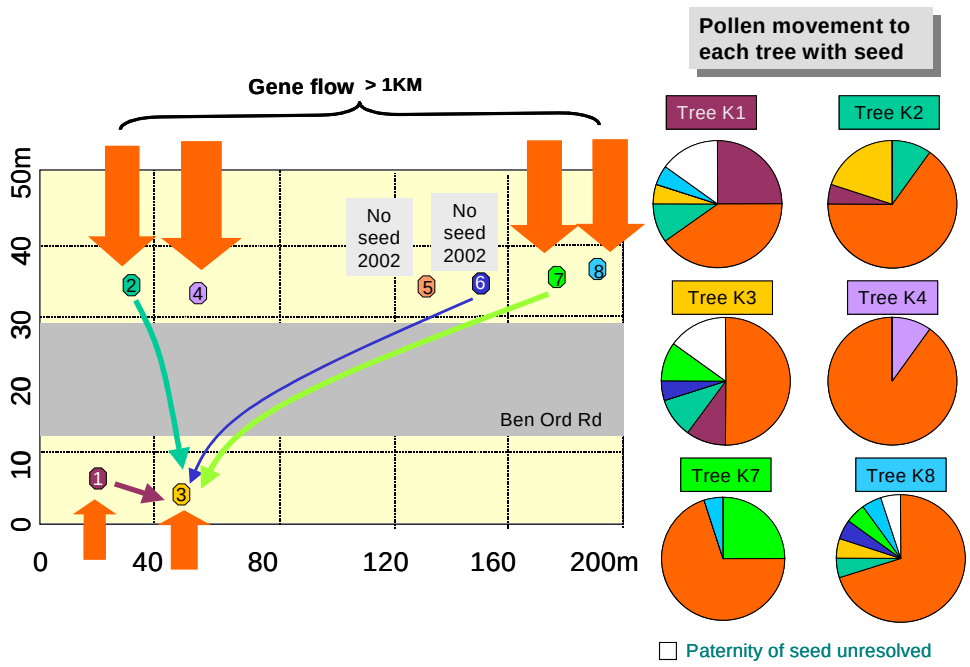
+ ≤ 0.1, ++ ≤ 0.05 positive effect.
- ≤ 0.1, -- ≤ 0.05 negative effect.

3.2 Landscape-level gene flow and genetic rescue in *Eucalyptus wandoo*

In addition to the general analysis of genetic diversity and inbreeding, the high-resolution SSR markers used *for E. wandoo* allowed direct assessment of the amount of gene flow into small populations by estimating the paternity of seed sired by local trees vs trees in other populations. The results indicate that significant proportions of seed in small populations are the result of fertilization by pollen from trees outside of their populations, with gene flow events being observed across landscapes at scales of several kilometres (Fig. 2 and see Appendix 7.4 Coates *et al.*).

This is a particularly important finding as it suggests that although the general genetic analysis indicated a strong relationship between population size and genetic variation in *E. wandoo*, it is clear that small populations would maintain even less diversity if they were not being genetically augmented by pollen-mediated gene flow from other local populations. This appears to be a clear example of the importance of landscape-level gene flow (interpopulation connectedness) facilitating “genetic rescue” of small populations. Data on the scale, amount and frequency of landscape-level gene flow and its importance for maintaining the viability of fragmented populations systems simply do not exist.

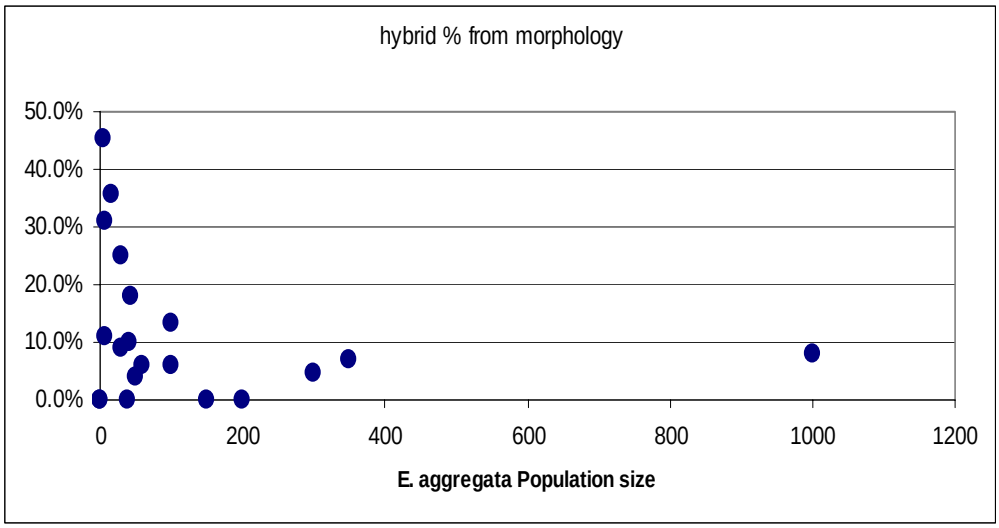
***Figure 2**. Paternity analysis for Eucalyptus wandoo showing significant gene flow into a small remnant population over scales >1km. Orange pie slices and arrows indicate immigrants*



3.3 Elevated hybridization in *Eucalyptus aggregata*

A second unexpected result came from the genetic analysis of the seed crop in *E. aggregata*. Like most of the species studied, *E. aggregata* showed reduction in genetic variation in small remnant populations, but little change in overall outcrossing rates, despite changes in reproductive sizes of several orders of magnitude (Table 4 & Appendix 7.2). However, the morphology of seedlings grown from seed crops from small populations showed dramatically increased morphological variation for a range of leaf and growth form characters. Subsequently, allozyme markers that can differentiation closely related eucalypt species were used to conduct multilocus assignment tests on 1200 of the seedlings measured for morphology, plus an additional 1200 seedlings sourced from the same seed crops. This analysis clearly showed significant increases in the production of *E. aggregata-E. rubida* and *E. aggregata-E. viminalis* hybrids as populations size got smaller, with populations less than 100 reproductive individuals having as much as 40% hybrid seed crop. In contrast to *E. wandoo*, this represents a situation where altered patterns of gene flow, in this case interspecific gene flow owing to landscape fragmentation, are having a deleterious effect on the genetic integrity of a species. This effect has not been previously observed, but given the propensity of eucalypts to hybridize, could well be a common genetic outcome in Australian landscape.

Figure 3. *Hybrid production increases in small Eucalyptus aggregata populations*



3.4 Geographical genetic structure in *Acacia acinacea* and *Swainsona sericea*

The data gathered for analysis of levels of genetic variation and inbreeding also facilitated measurement of interpopulation genetic differentiation. For five of the seven target taxa levels of genetic relatedness among populations were within expectations for plant species distributed across 10s to 100s of km. However, highly significant genetic differentiation was observed across the range of two of the target taxa, *A. acinacea* and *S. sericea*. Subsequent morphological and cytogenetic analysis showed that both of these species exhibit cryptic chromosomal variation, with populations displaying several chromosome numbers (*A. acinacea* 2n=26, 52, *S. sericea* 2n=32, 80 and 128). Knowledge of these gross genetic differences among populations within landscapes is critical as mixing of individuals either as result of changes in landscape level gene flow (as evidenced for *E. aggregata* and *E. wandoo*) or through bulking of seed collections for revegetation work, will result in the inadvertent production of triploid hybrids and the subsequent proliferation of backcrossed aneuploids. Such chromosomal changes are highly undesirable, usually being associated with significant reductions in fitness. Little if anything is known about the extent and geographical scale of cytogenetic complexity of the native plant species commonly being used for revegetation and restoration activities in Australia.

Another target taxa, *Chrysocephalum apiculatum*, was abandoned in the first year of this study when it too was found to be polyploidy (tetraploid) as at that point it was thought that polyploidy was not likely to be representative of most species in the study biomes. In the grassland-woodland biome in the south east this assumption appears not to be true.

3.5 Pollination, fecundity and fitness

While genetic parameters are important indicators of population health, and reflect changes in both genetic and ecological or demographic dynamics, population viability is most directly affected by those ecological shifts resulting from habitat fragmentation that influence reproduction and fitness. Some of these will be influenced by genetics such as effects of inbreeding on fitness, or genetic erosion on mate availability in self-

incompatible species (Les *et al.* 1991; Buza *et al.* 2000; Young *et al.* 2000). Others will be purely ecological, such as the loss or change in composition and abundance of pollinator guilds in degraded sites (Lamont *et al.* 1993) affecting patterns of pollination and seed set.

Table 5 and Appendix 7.3 summarize the relationships observed between population and landscape parameters and the direct measures of fecundity and fitness that were obtained for five of the seven target taxa. These studies were undertaken on a subset of up to 16 of the populations sampled for the genetic analyses.

Table 5 Effect of population size, isolation and disturbance on pollination, fecundity and seedling fitness

Taxa	Population landscape parameter	Flowers	Pollen visits	Pollination	Seed set	Seed germ	Growth
A. dealbata	Size	+		ns	ns	ns	++
	Isolation	ns		++	ns	ns	--
	Disturbance	ns		ns	ns	ns	+
A. acinacea	Size					ns	ns
	Isolation					ns	++
	Disturbance					ns	ns
S. sericea	Size	ns			ns	-	++
	Isolation	+++			--	ns	-
	Disturbance	ns			ns	ns	-
E. wandoo	Size			--	++	--	ns
	Isolation			ns	ns	ns	ns
	Disturbance			ns	ns	ns	ns
C. quadrifidus	Size		+++	ns	+++	ns	ns
	Isolation		ns	--	ns	++	ns
	Disturbance		ns	ns	ns	ns	ns

+ ≤ 0.1, ++ ≤ 0.05, +++ ≤ 0.001 positive effect.
- ≤ 0.1, -- ≤ 0.05, --- ≤ 0.001 negative effect.

As with the genetic response variables, the reproductive population size at a site is the primary driver of these ecological responses to ecosystem fragmentation, with several of the species displaying relationships between size and measures of both fecundity and, importantly, fitness. Isolation also appears important, but generally affects fitness parameters more than fecundity. Interestingly, there is a differential response between the two landscapes. Target taxa from the fragmented kwongan biome, with its clear cut differences between remnant vegetation and non-native inter-remnant matrix, primarily exhibit changes in fecundity with isolation and size. Those from the variegated grassland-woodland biome, with its more subtle grades of isolation among remnants, appear to respond more in terms of fitness parameters – seed germination and seedling growth.

4. CONCLUSIONS

Key objectives of this research project were to, through comprehensive genetic and ecological analysis of a range of target taxa: 1) identify genetic and demographic effects of ecosystem fragmentation; 2) determine how these effects relate to population and landscape parameters; 3) investigate the influence of species life-history traits on these responses and, based on this; 4) generate landscape design principles that will minimise these effects and so maximize population viability and the probability of long-term species persistence within landscapes.

These have proved to be challenging tasks, particularly because of the temporal limitation of the sampling (two field seasons), the relatively small number of taxa that have been surveyed due to the intensive nature of the genetic analysis component of the project, and the diversity of responses observed among these species. Nevertheless, the results obtained represent the first data set of its kind, and provide several important insights in to these issues from which some genetic and ecological landscape management principles can be drawn.

4.1 Genetic and ecological effects of ecosystem fragmentation are real for common species. Negative genetic and demographic changes do occur in populations of common native plant species in fragmented landscapes that are likely to effect both local population viability and the utility of these populations as seed source for revegetation and restoration activities. This supports previous data from rare plants and confirms landscape fragmentation as a major threatening process for Australian plant ecosystems.

4.2 Population size is critically important. Reproductive population size is the primary population/landscape variable underpinning changes in measured genetic and reproductive parameters. Isolation plays a secondary, but significant, role in determining fecundity and fitness but has less effect on genetic variation. Clear effects of disturbance on either genetic or demographic processes are not evident.

The nature of the relationship between size/isolation and population performance or "health" is that smaller more isolated populations may perform well or poorly in terms of the genetic and demographic variables assayed, while large populations perform consistently well. Main effects observed in small populations include:

i. Reduced pollinator service
ii. Reduced fertilisation and seed set
iii. Loss of genetic variation – "genetic erosion"
iv. Increased inbreeding via a combination of outcrossing and biparental inbreeding.
v. Reduced effective size of paternal pollen pools. This is critical as increases in correlation of parentage priming populations for biparental inbreeding in subsequent generations.
vi. Lower fitness as measured by seed germination and seedling growth.

4.3 Species have variable response profiles. None of the target taxa showed all of the genetic and ecological effects described above. However, nor are there any obvious relationships between species life-history traits and responses. This makes it very difficult based on these data to generalise as to the relative threat status of different types of species. It is not clear whether this is because life history traits don't have an effect on response to fragmentation, or whether this study did not have the power to isolate these effects due to low replication at the life-history level. However, there is evidence for differences in the biological responses of the species to the fragmented landscape of the kwongan shrublands in Western Australia and the variegated grassland-woodland communities in New South Wales, with more effect of population size, and particularly isolation, on fitness parameters observed in the grassland-woodland ecosystem.

4.4 Viability thresholds are in the 100s of plants. Most positive relationships observed between size and genetic and demographic variables were log-linear in nature and suggest that, across species, major negative effects were encountered in

populations smaller than 100-200 reproductive plants. Though a coarse measure this represents best current knowledge of minimum population sizes that should consistently exhibit good genetic and reproductive characteristics. Many smaller populations will perform well, but many will also perform poorly and this is not readily predictable.

4.5 Hybridization is important. In addition to genetic erosion and inbreeding, increased hybridization with reduced population size has been identified as an major threat to the genetic integrity of plant species in small remnants. This is likely to be a widespread issue for several important Australia plant groups such as the eucalypts in which interspecific mating barriers can be weak.

4.6 Interpopulation gene flow is critical. Data from *E. wandoo*, the only species for which markers were available to undertake direct assessment of interpopulation gene flow, clearly indicate that small populations can be are genetically rescued by inputs from other remnants in the landscape over scales of up to several kilometres. This phenomenon may explain some of the variability in response observed among small populations of other species, and the observed influence of isolation on seed set in *S. sericea*. These results reinforce the importance of managing landscapes rather than individual populations.

A quantitative understanding of the scale, extent and temporal dynamics of interpopulation gene flow in fragmented landscapes, combined with a more sophisticated approach to understanding biologically relevant landscape structure, is likely to be important in explaining the variation in population and species responses highlighted by the current data.

4.7 Strong interpopulation genetic structure exists. There is a surprisingly high degree of complexity and geographical structure of genetic variation among populations of some species. The influence of this structure on population viability and conservation utility is particularly important when variation is for gross cytogenetic differences among populations within landscapes, such as those identified for *A. acinacea* and *S. sericea* in the south east. Such chromosomal differences have direct and strong effects on population fitness should they be mixed, or linked by gene flow owing to the dramatic effects of dysgenesis on fitness.

5. LANDSCAPE MANAGEMENT PRINCIPLES

The final objective of this project was to combine the data on ecological and genetic responses, with the landscape data across species and biomes, to produce general landscape design principles that can be used to guide the management of fragmented ecosystems for long-term integrated conservation of remnant native vegetation.

It is clear from the results presented that there is much still to be done in terms of understanding the genetic and ecological responses of Australian native plants to ecosystem fragmentation. This is especially true with regard to the importance of among population gene flow in maintaining population function, the implications of observed genetic changes for long-term viability and the influence of life-history traits on species sensitivity to landscape change. Nevertheless, based on the data set from these seven target taxa, it is possible to make several fairly clear recommendations that are broadly applicable to the maintenance of both the ecological and genetic viability of native plants species for both of the landscapes under consideration:

1. Maintain populations larger than 100-200 reproductive plants when possible. Such populations are critical components of fragmented landscapes because they perform significantly better in terms of reproductive output and have greater genetic diversity and less inbreeding than smaller populations.
2. Minimise isolation distance among populations to maintain biological connectedness through pollen flow and seed dispersal. While population size was consistently the key parameter associated with changes in genetic and demographic processes, isolation also had an influence on pollination, reproductive output and fitness. Connectedness may be facilitated by improvement of the inter-remnant matrix as well. In *E. wandoo* for instance, the maintenance of paddock trees that act as stepping stones for pollinators is likely to be important in maintaining gene flow among populations.
3. Site condition is not as important as population size or remnant isolation in determining genetic and demographic performance and should be given less consideration in landscape planning.
4. Manage populations within landscapes together over scales of 5-20 km and not as a series of populations independent of other vegetation in the area.

There are a range of critical knowledge gaps in our present understanding of how ecosystem fragmentation influences the viability of remnant plant populations through its effects on ecological and genetic processes. Four key issues stand out as being very important and yet being very poorly understood:

1. The frequency, extent and scale of genetic and demographic connectedness among populations.
2. The influence of life-history traits or ecological “type” on sensitivity to fragmentation.
3. The medium-term effects of accumulated inbreeding through biparental mating.
4. The importance of geographical genetic structure within landscapes for adaptive traits and for gross cytogenetic differences.

Greater knowledge in these areas is likely to lead to significant improvement in our ability to effectively manage Australia’s fragmented native ecosystems for long-term conservation.

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7. APPENDICES

7.1 Target taxa descriptions and distributions

7.2 Plots of population size vs genetic diversity, inbreeding and correlated paternity

7.3 Plots of population size vs fecundity and fitness

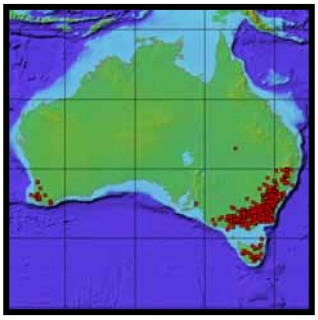
7.4 Scientific Papers

7.5 Communications achievements

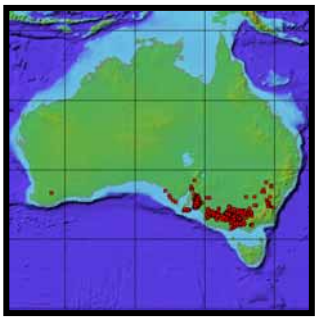
7.6 Streamline abstract

Appendix 7.I: Target taxa descriptions and distributions

Acacia dealbata (tree)



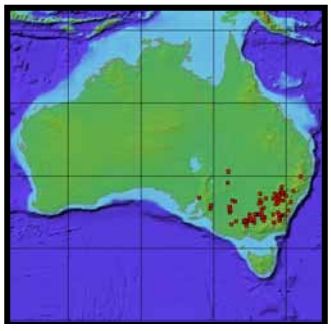
Acacia dealbata (Mimosaceae), commonly known as the silver wattle, is a small tree to 5m with silvery green to dark green fern-like leaves. From late winter to spring the species produces a mass of inflorescences each comprised of 25-35 golden yellow flowers. It can also reproduce asexually through suckers and can readily regenerate following disturbances such as fire to form thickets. These thickets have been reported to provide important habitats for some bird and animal species. *Acacia dealbata* is a common and widespread component of the grassy woodlands.



Acacia acinacea (shrub)

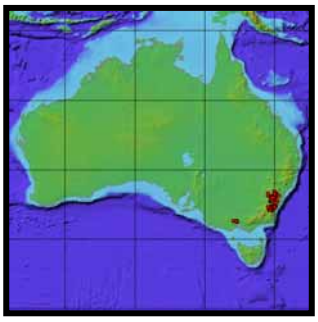
Acacia acinacea (Mimosaceae), commonly know as the Gold-dust wattle, is widely distributed across South Australia, Victoria and New South Wales. It is a small to medium bushy shrub to 2.5 m high and grows mostly on sand, sandy loam and gravelly soil. It is found in a variety of habitats such as hilly country, eucalypt woodland, woodland heath and open mallee scrubland. Morphologically it is highly polymorphic and several growth forms are known. Bright yellow inflorescences occur in spring and legumes are twisted or coiled.

Swainsona sericea (herb)

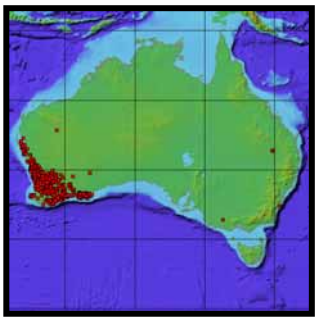


Swainsona sericea (Fabaceae) is a small prostrate to erect herb up to 10 cm tall with hairy grey green leaves and purple pea-like flowers. The flowers are produced in groups of 2 to 8 at the end of flower stems from October to December. It can also regenerate after fire from seed stored in the seed soil bank. It is moderately common in the grasslands and grassy woodlands of the southern tablelands of NSW and the ACT but is more likely to be found on less disturbed sites.

***Eucalyptus aggregata* (tree)**



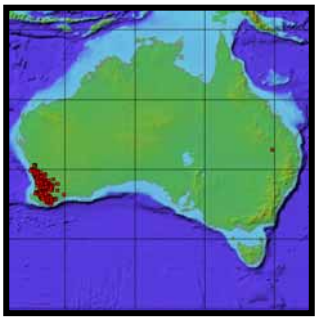
Eucalyptus aggregata (Myrtaceae) is commonly known as black gum and is a small to medium sized tree to 20m. It has rough flaky dark green to grey green bark which persists to the small branches. The adult lanceolate shaped leaves are petiolate and alternate and slightly glossy to dullish with moderate reticulation. Inflorescences are produced from December to February and occur as are umbels of seven white or cream shortly pedicellate flowers. It belongs to the small fruited swamp gums and prefers moist sites such as swamps and poorly drained hollows areas making it somewhat uncommon.



***Calothamnus quadrifidus* (shrub)**

Calothamnus quadrifidis is a widespread erect compact shrub 2-3 m high that grows on a wide variety of soil types and habitats throughout the High and Transitional Rainfall Zones of south-west Western Australia. The species has inflorescences which are cauliferous each comprising of 10-30 flowers that are generally pendulous and borne on one side of the stem. Flowers are predominantly protandrous and are pollinated mainly by honeyeaters.

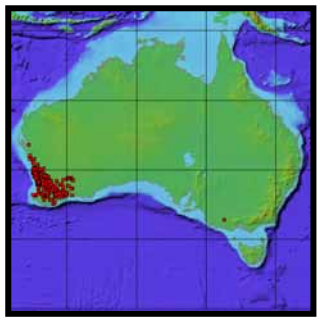
***Eucalyptus wandoo* (tree)**



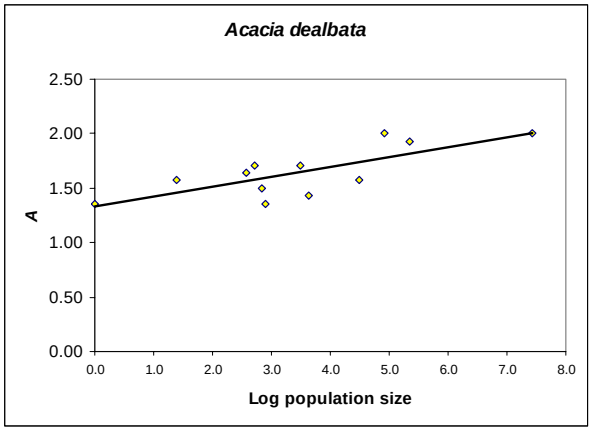
Eucalyptus wandoo is a widespread small to medium sized tree 3-25m high that grows on a wide variety of soil types on the eastern side of the High Rainfall Zone and western side of the Transitional Rainfall Zone of the south-west Western Australia (for distribution map see attachment 6d). The species is mass flowering, cream white flowers are arranged in more than 7-flowered unbranched inflorescences borne in leaf axils on the outer part of the tree canopy. It is insect- pollinated and wind-dispersed.

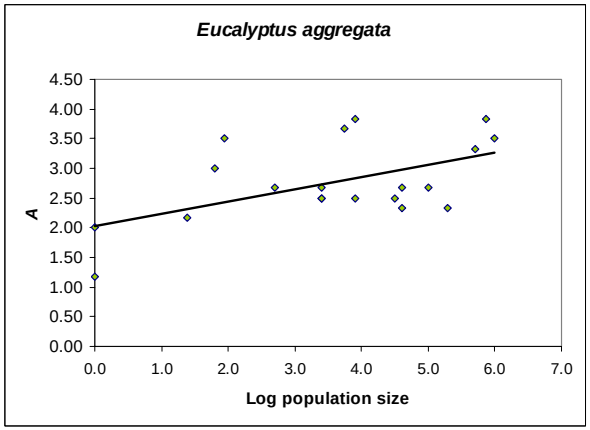
***Eremaea pauciflora* (shrub)**

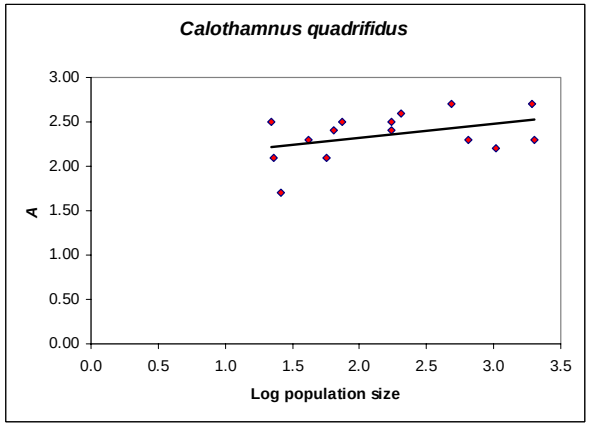
Eremaea pauciflora is a widespread small to large spreading woody shrub 0.5 to 2m high growing largely in deep sandy soils and sand over laterite. It is distributed throughout a significant area of the south-west Botanical Province of Western Australia. The species is generally sparsely flowering with orange flowers, borne mostly singly at the ends of long branches.

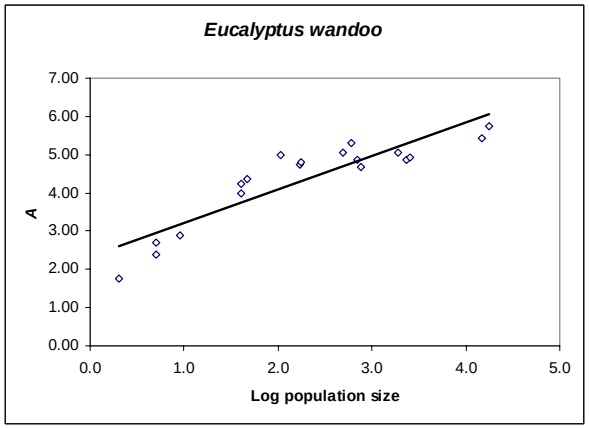


Appendix 7.2: Plots of population size vs genetic diversity, inbreeding and correlated paternity

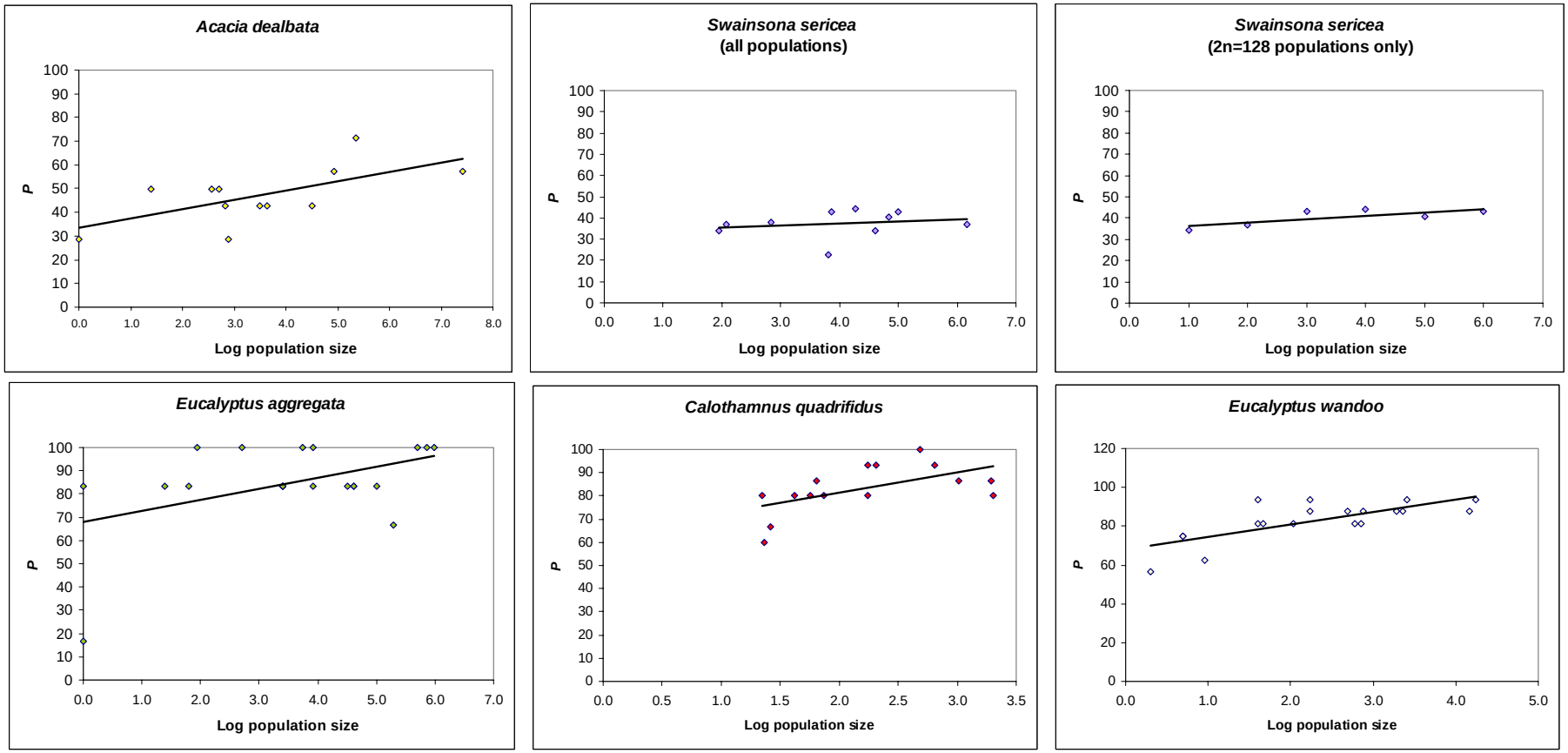




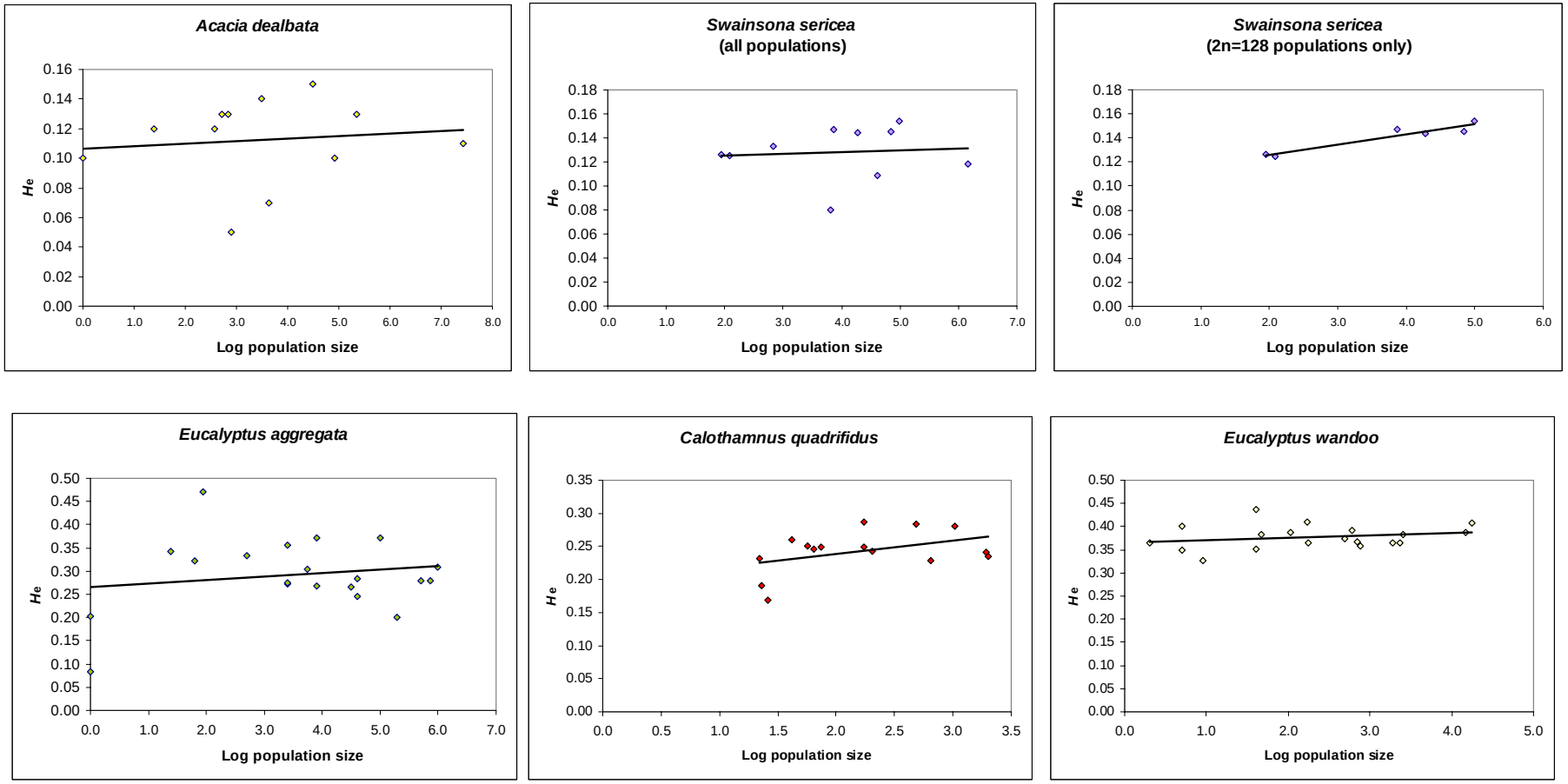




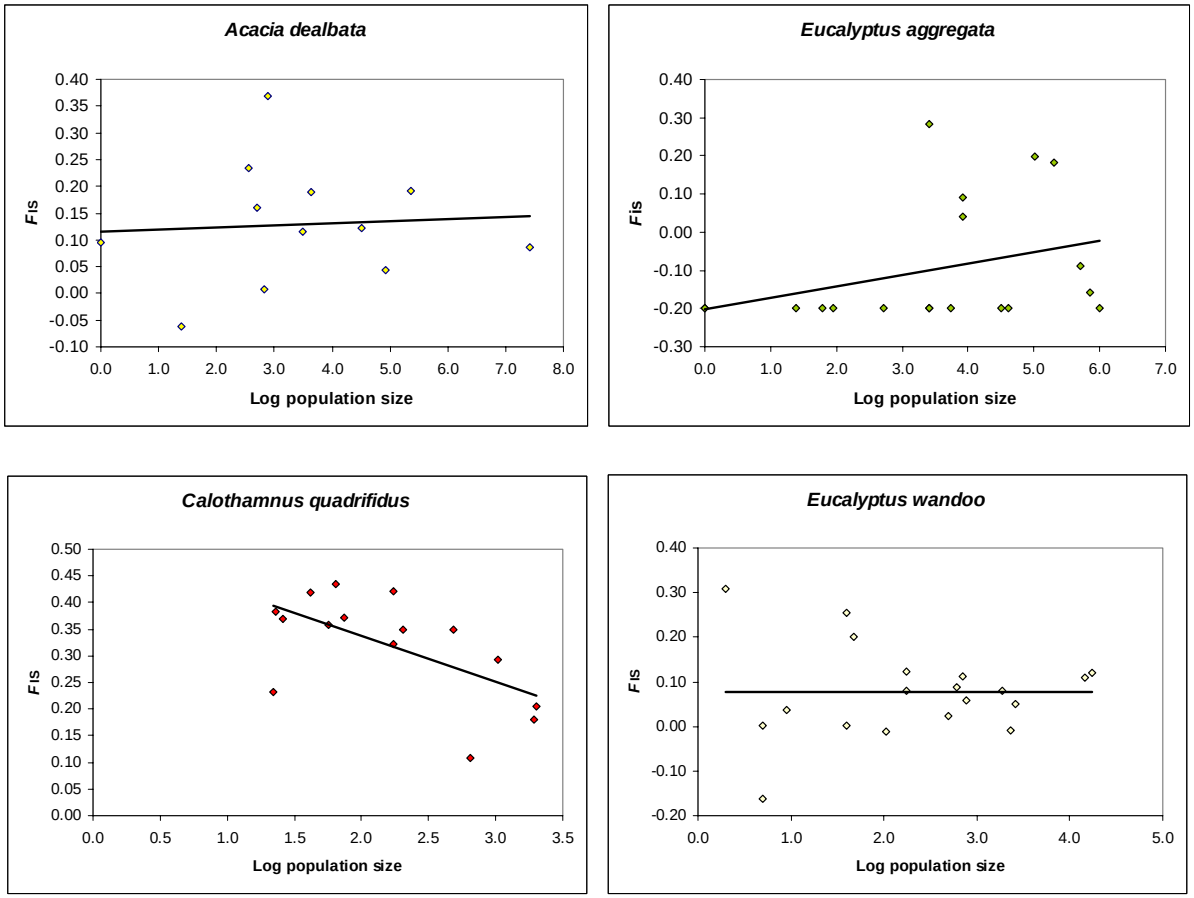
Allelic richness for target taxa.



Percentage of polymorphic loci for target taxa.

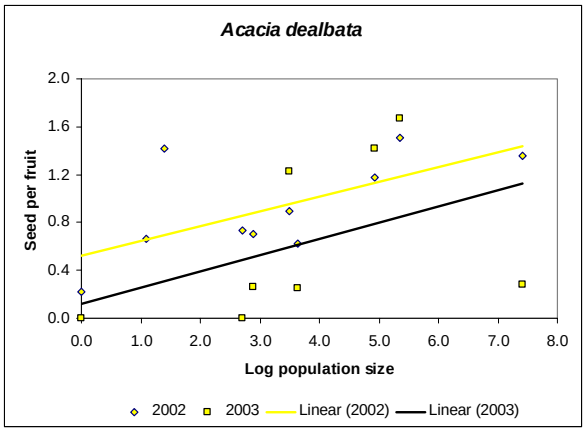


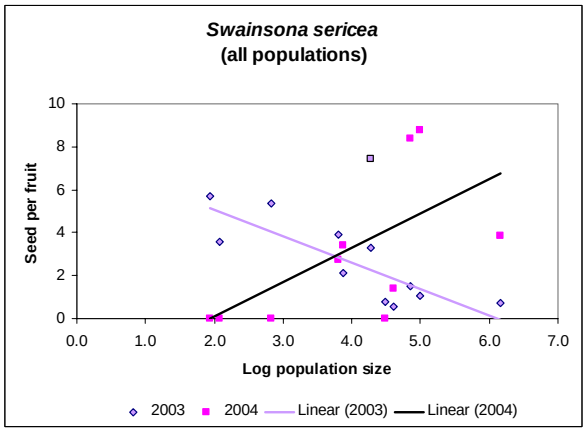
Gene diversity for target taxa.

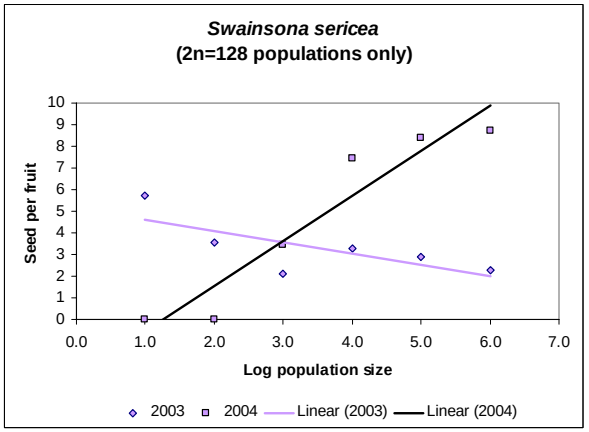


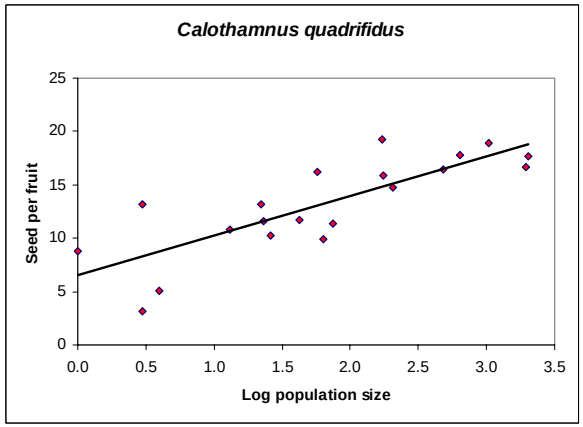
Fixation coefficient for target taxa.

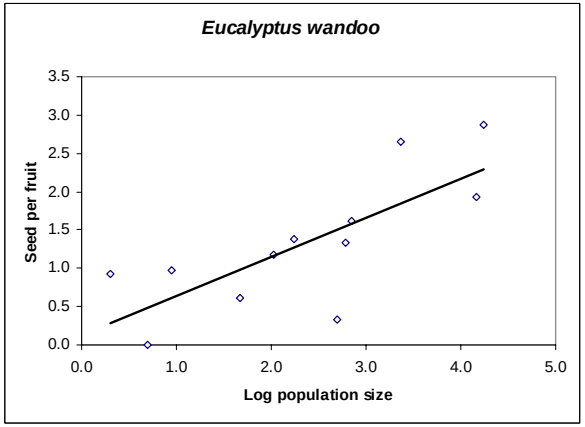
Appendix 7.3: Plots of population size vs fecundity and fitness



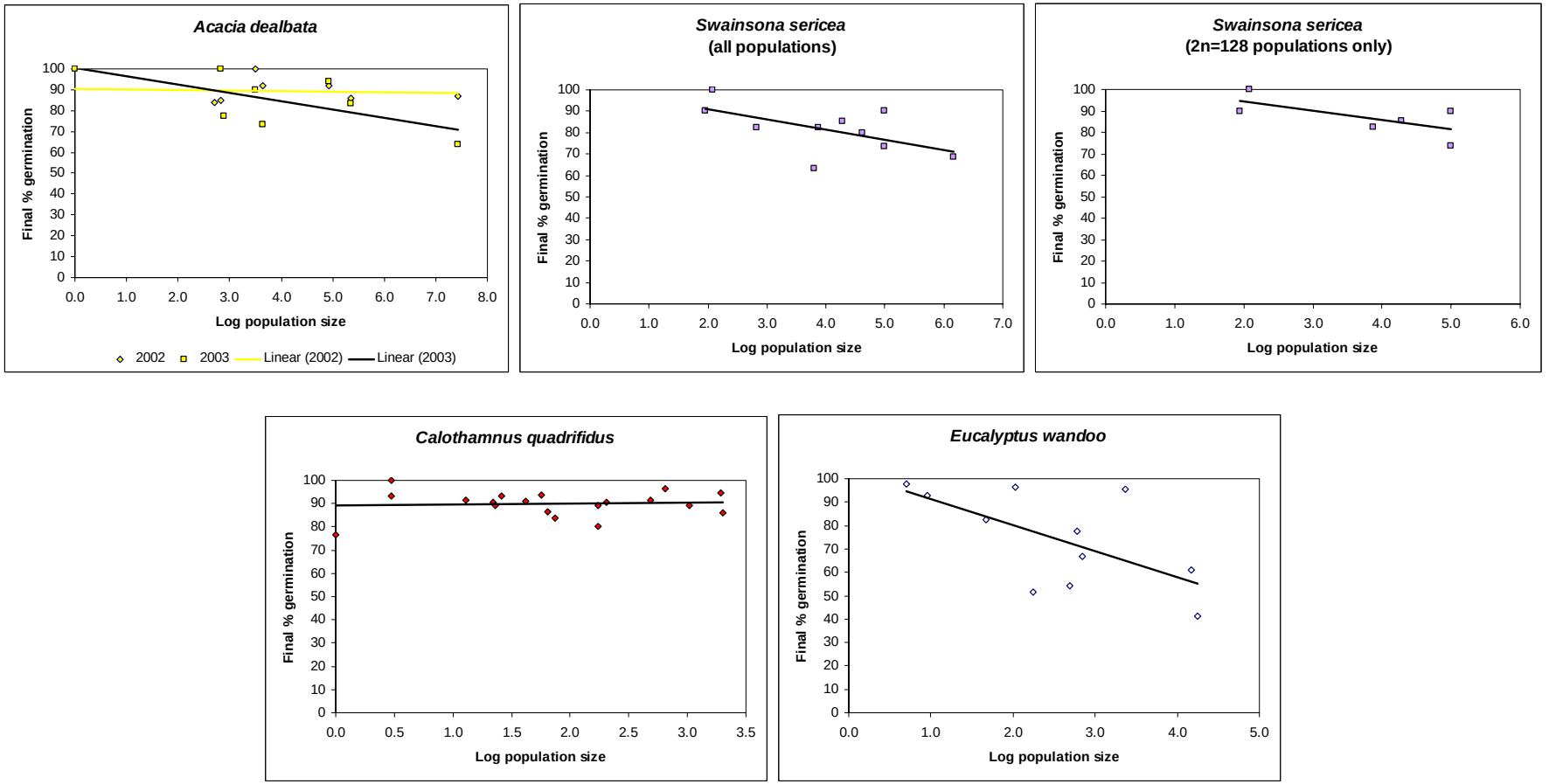




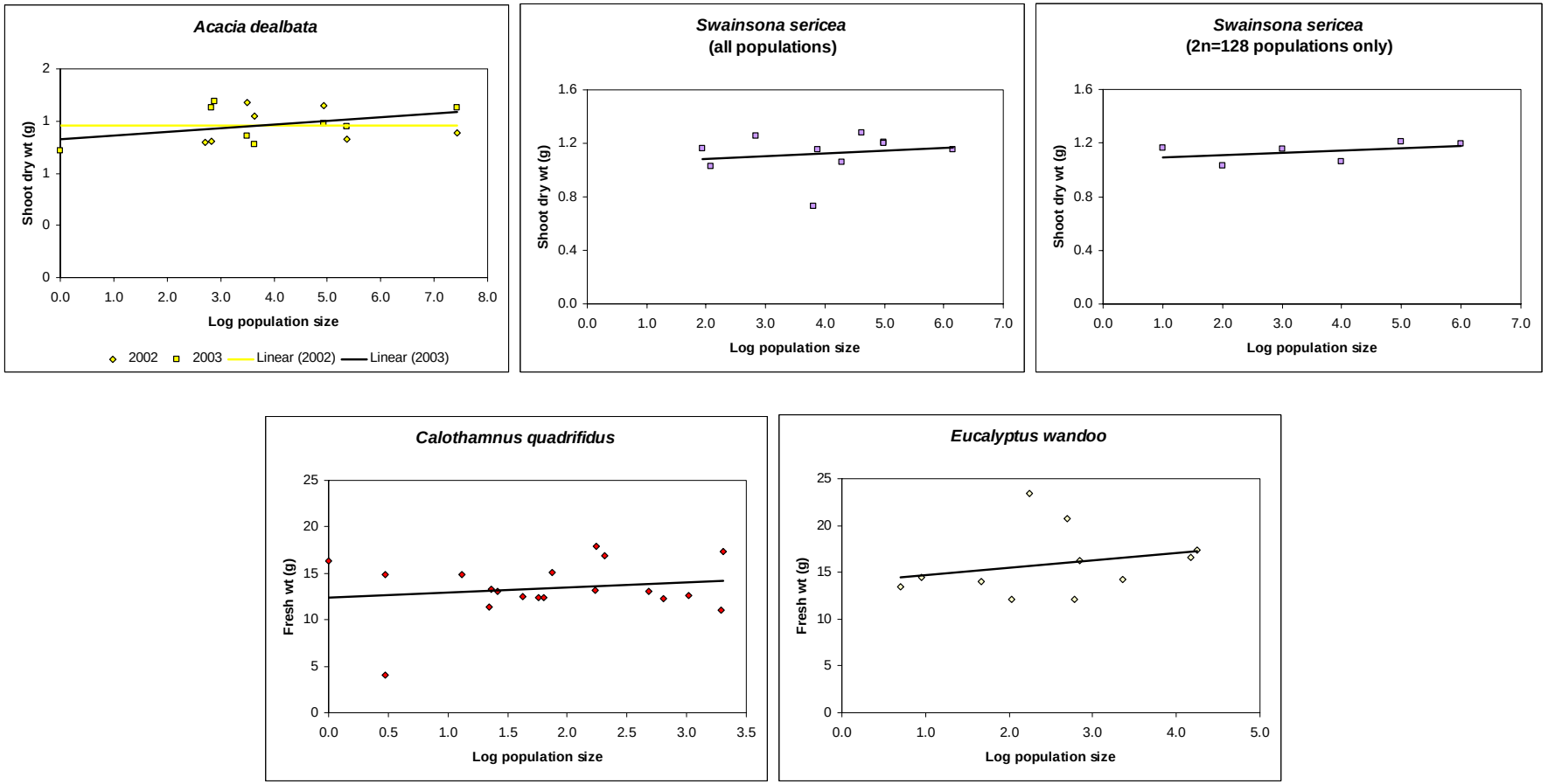




Mean seed set per fruit for target taxa.



Final percentage germination for target taxa.



Fitness trial mean seedling weight for target taxa.

APPENDIX 7.4: Scientific Papers

Broadhurst L.M., Young A.G., Thrall P.H. and Murray B.G. (in press) Sourcing seed for *Acacia acinacea*, a key revegetation species in south eastern Australia. *Conservation Genetics*.

Coates D. J., Byrne M., Elliott C., Sampson J. and Yates C. (in press) Gene flow and outcrossing in a fragmented landscape: implications for seed sourcing from small remnants. In *Proceedings of the Fifth Australian Workshop on Native Seed Biology*. Eds Adkins, S., Bellairs, S. and Coates D. Australian Centre for Minesite Rehabilitation. Brisbane.

Elliott, C and Byrne, M. (in press) Isolation and characterisation of microsatellites in the woody shrub, Calothamnus quadrifidus (Myrtaceae). *Molecular Ecology.*

Young A.G. (in press) "Non-provenance genetic issues for native seed sourcing". In *Proceedings of the Fifth Australian Workshop on Native Seed Biology*. Eds Adkins, S., Bellairs, S. and Coates D. Australian Centre for Minesite Rehabilitation. Brisbane.

Colin J. Yates, David J. Coates, Carole Elliott And Margaret Byrne (submitted) Bird pollination and mating system variation for a shrub in fragments of species rich kwongan in south-west Western Australia. *Biodiversity and Conservation.*

Sourcing seed for *Acacia acinacea*, a key revegetation species in south eastern Australia

Running title: Seed sourcing in *Acacia acinacea*

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Keywords: seed sourcing, revegetation, morphology, AFLP, ploidy

Received

Abstract

Intensive large-scale revegetation programs are a major activity in degraded landscapes. The collection and deployment of seed for revegetation is primarily guided by sourcing locally to preclude concerns associated with local adaptation and outbreeding depression. For most species however, little is known about the levels of genetic diversity being sourced and deployed, or of the levels of adaptively significant variation present across the species range. *Acacia acinacea* (gold dust wattle) is a key revegetation species widely distributed across south eastern Australia. Levels of variation in terms of morphology, fitness, ploidy level and AFLPs were assessed using seed lots from 35 populations across the Murray Darling Basin in south eastern Australia. Multivariate analysis indicated that three of the populations were misidentified while the remaining populations were differentiated into three groups. One of these groups (Bendigo) was highly geographically localised and tetraploid while the other two (Group1 and Group2) were both diploid with overlapping distributions. Fitness differences were also evident among these three groups while AFLPs indicated that they were genetically differentiated and that lower levels of diversity were captured from some populations. A major finding of this study was that no one technique was able to fully describe the variation present in *A. acinacea* and seed sourcing guidelines based on any single approach could have resulted in erroneous decisions being made. Information generated from this study has been used to refine seed sourcing guidelines.

Introduction

Like many other regions in the world, degraded Australian landscapes are now the focus of intensive large-scale restoration programs. From modest beginnings in the late 1980s revegetation efforts have grown rapidly in recent years in response to the increasing environmental consequences associated with land clearing. Seed collection for revegetation in Australia is primarily guided by the perceived requirement to source locally to preclude concerns associated with local adaptation and outbreeding depression. These effects have been demonstrated in several plant species although heterosis can initially mask outbreeding depression which may only become obvious in subsequent generations (Fenster & Galloway 2000, Keller et al. 2000, Montalvo & Ellstrand 2001, Quilichini et al. 2001). Two mechanisms are thought to be responsible for this reduction in fitness - the dilution of locally adapted genes and the break-up of co-adapted gene complexes (Templeton 1986, Fenster & Dudash 1994). Regardless of which mechanism is involved, both have the potential to reduce the success of revegetation efforts and to jeopardise the long-term persistence of local populations. The utilisation of local seed also presents another often overlooked advantage, namely the synergy between locally adapted populations and the organisms with which they interact. For example, bud burst in local *Crataegus monogyna* plants coincides with the emergence of many Lepidopteran species for which this species is an important food source whereas bud-burst in non-local plants occurs much later (Jones et al. 2001). Such interactions are pivotal to revegetation success since the objective of most replantings is to establish self-sustaining communities which includes the establishment and maintenance of interactions with organisms such as pollinators, predators and soil symbionts similar to those found in natural communities.

While utilising local seed has many advantages, in practice this requirement can also be so restrictive that a lack of local seed sources has resulted in the abandonment of some revegetation programs (Wilkinson 2001). This is of particular

concern in highly degraded landscapes where little natural vegetation remains. In addition, seed in these regions is often sourced from small and highly fragmented remnants which, depending on the reproductive strategy of the species in question, can be severely compromised by genetic erosion and inbreeding (Prober & Brown 1994, Young et al. 2000, Tomimatsu & Ohara 2003). The demographic consequences of this include poor seed set, smaller seed, slower germination response, poor seedling survival and reduced reproductive capacity (Buza et al. 2000, Oostermeijer 2000, Young et al. 2000, Tomimatsu & Ohara 2003). Under these circumstances revegetation practitioners are faced with a difficult choice. On the one hand they can deploy poor quality local seed with a narrow genetic base that may compromise both the immediate success of revegetation due to germination failure and poor survival and its longer term evolutionary potential (Gordon & Rice 1998). Alternatively, practioners can source seed from more distant sites that exhibit superior innate fitness, but which may compromise revegetation success through poor adaptation as well as the genetic integrity and viability of small local populations through outbreeding depression. Understanding this trade-off between maintaining local adaptation to enhance fitness, and possible reductions in fitness owing to genetic erosion and inbreeding is critical for land managers to make the most appropriate decisions regarding their seed sources.

Despite the considerable resources currently being directed towards revegetation, almost nothing is known about the levels of genetic diversity being sourced and subsequently deployed, or of the levels of population differentiation within species. Populations with greater genetic variation are generally considered to be better sources of seed for revegetation since these presumably have increased fitness and can meet future adaptive requirements (Helenurm 1998), while genetic differentiation at the population level may provide some indication of the distances over which seed can be moved without impacting revegetation success or local populations owing to poor adaptation. This is particularly critical for practitioners in regions where

little natural vegetation remains and the majority of seed is sourced from remnant vegetation that is small, isolated and/or degraded. Seed collecting may also be compromised by other issues. For example, many widespread species targeted for broadscale revegetation projects also exhibit morphological variation across their distribution and it is often unclear whether this variability represents phenotypic plasticity, adaptively significant quantitative traits that should be restricted to the local environment or, owing to the paucity of taxonomic scrutiny, represent new taxa not formally described.

Acacia acinacea (Fabaceae: Mimosoideae), commonly known as the Gold Dust wattle, is a key species for revegetation in south eastern Australia. It is widely distributed across South Australia, Victoria and New South Wales and part of this range overlaps with its close relative *A. microcarpa*. Two other close relatives, *A. imbricata* and *A. triquerta*, are restricted to the area around Pt Lincoln in South Australia (Maslin 1987). Morphological variability is known from across the range of *A. acinacea* (Maslin 2001) although the distribution and extent of this variation is unclear, and in the absence of a formal taxonomic treatment revegetation practitioners currently manage *A. acinacea* as a single entity. Under this premise there is considerable potential to deploy germplasm inappropriately. This study was primarily interested in examining *A. acinacea* seed being sourced by Greening Australia, one of the major revegetation agencies in south eastern Australia, from across it's range within the Murray Darling Basin to determine the levels of quantitative and genetic variability currently being deployed during revegetation. Accordingly, we assessed morphological variation, ploidy level, fitness and genetic diversity of 35 bulk seed lots from natural populations. We were also interested to ascertain whether this comprehensive approach could identify populations with atypical traits that might indicate local adaptation. Our objectives for this study were to 1. examine variability within *Acacia acinacea* using a range of techniques, 2. determine the efficacy of each technique to

quantify variability, and 3. use our findings to refine current revegetation activities.

Methods

Seed collection and growth trial

Bulk seed collections and collection co-ordinates from 35 populations distributed throughout the Murray Darling Basin (Fig. 1) collected under Greening Australia (GA) guidelines were provided by four regional seed bank co-ordinators from stock supplies. These guidelines recommend that seed be collected from as many healthy plants distributed across natural populations as possible. Most populations were collected during summer 2001-02 although three collections were from 1999 (Moliagul, Bromley and Whume99) and one from 1995 (Whume95). Seed was maintained under low temperature storage conditions (4^{o}C) and is regularly tested for viability. All seed was sourced from wild plants with the exception of West Hume (WHume95) which is a seed orchard. To ensure anonymity throughout the study, collections were known by individual codes only. Forty seed from each population were weighed, surface sterilized and the testa nicked, soaked overnight and sown directly into 8 cm pots containing a 1:1:1 mixture of peat moss, sand and compost using an unbalanced randomised block design with position randomised for population (35) and block (6) for the 1400 individuals. Pots were maintained in a heated greenhouse at 25^{o}C under natural light and emergence and survival were checked weekly until harvest (20 weeks) with emergence being defined as when the cotyledons emerged 5 mm from the soil. As the trial progressed an unexpected level of morphological variability became apparent and, prior to harvest, several quantitative measures were recorded (see *Quantitative variation* below). At harvest plant height was recorded by measuring the longest stem and plants were then divided into root and shoot components, the roots washed and both components dried and weighed. Both Currency (7) and Nairne (9) were eliminated from most statistical analyses owing to germination failure but where data exist, these

are provided for information purposes. All experimental assessments were done blind to prevent unintentional bias. Unless stated otherwise, all data analysis was conducted using GENSTAT version 7 (GenStat 2003).

Quantitative variation

The level of morphological variation present in the growth trial indicated that plant form might have an important influence on interpretation of the fitness and genetic data. Six semi-quantitative measures were assessed prior to harvest and analysed to determine whether coherent morphological groups could be identified. The traits assessed were leaf shape (1 = oval, 2 = midshape, 3 = elongate), hairs (presence/absence), the extent of apical (Fig. 3d) and basal (Fig. 3e) stem branching (0 = no stems, 1 = <10 stems, 2 = 10+ stems), the retention of juvenile foliage (0 = none, 1 = <10, 2 = ≥10 phyllodes) and whether stems were ridged (0 = none, 0.5 = weak, 1 = ridged). Plant height was corrected to remove any bias associated with date of germination by dividing height by the number of days since emergence. Mean trait values were calculated for each population and a principal component analysis (PCA) conducted using a correlation matrix to standardise variates since metrics did not have a common scale and showed different amounts of variation (GenStat 2003). Once the appropriate number of components had been determined, varimax rotation was applied to improve interpretability of the factor extraction (Tabachnick & Fidell 2001). This analysis indicated that the West Hume populations were highly differentiated from the remaining populations and these were removed and the analysis re-run. To remove possible ploidy level effects, a further PCA analysis was conducted to assess differentiation among those populations with n=26 chromosomes only which removed Pt Lincoln (8) from Group1. All further analyses which refer to Group1 include n=26 chromosome populations only.

Ploidy level

Ploidy level was assessed since evidence of polyploidy exists in other *Acacia* species (Blakesley et al. 2002, Miller et al. 2002) and, if present, would influence interpretation of the fitness and genetic data. Seed (approx. 40) from each population were sown into individual trays with the growth trial potting mix and chromosome numbers were determined from mitotic cells obtained from actively growing root tips. Root tips were collected into 0.05% colchicine solution and kept at 4^{o}C for approximately 18h. These were then fixed in ethanol:glacial acetic acid (3:1) for 24h then transferred to 70% ethanol and stored at 4^{o}C. To observe the chromosomes, root tips were hydrolyzed in 1*M* HCl at 60^{o}C for 8min, then transferred to 45% acetic acid and finally macerated in a drop of FLP orcein (Jackson 1973) on a microscope slide. A coverslip was added, the material was gently heated over a spirit flame and squashed between layers of filter paper to flatten the cells. Five to 10 cells were observed from at least one plant from each population. Stomatal guard cell measurements were assessed from epidermal peels taken from the tenth phyllode from the apex of a young shoot and mounted in 50% silver nitrate on a slide. The length of ten guard cells was measured with a compound microscope with an eyepiece scale (graticule) using a x40 objective.

Fitness

REML analysis was used to assess whether differences existed among blocks in the number of emergents and survivors and the number of days taken to emerge (block = fixed, population = random) once the tests of assumptions of normality and equality of variance had indicated that no transformations were required. The Wald χ^2 statistic indicated that no differences among blocks existed for any of these variables so data were pooled into their respective populations and the final percentage emergence and survival were calculated as was the mean number of days to emergence following

removal of non-germinants. Associations between seed weight and these three variables were also assessed by linear regression.

Differences among blocks for the growth variables of plant height (Ht), root (Rt) and shoot (Sht) weight and the root to shoot ratio were also assessed once the assumptions of normality and equality of variance were checked and the root to shoot ratio arcsine transformed (ASINRt:Sht). REML analysis found significant block differences for all variables. To test whether these differences were associated with a particular block, means for populations nested within blocks were generated by REML and block differences assessed by one-way ANOVA using the population means within blocks as replicates. Differences among the block means were compared using Tukey's post hoc test. The REML-predicted population means for the growth variables were also used to test for differentiation among populations by one-way ANOVA using block means as replicates and means again compared using Tukeys post hoc test. Associations between seed weight and each of the growth variables were assessed by linear regression.

Since the Group1 (n=26 only), Group2 and Bendigo populations are the most collected and deployed seed sources for revegetation, fitness differences among and within these groups were assessed by one-way ANOVA using block means for each population as replicates with the means compared using Tukeys post hoc test. Linear relationships between seed weight and the fitness variables for each of the three taxa were also assessed.

DNA extraction, AFLP procedures and data analysis

Forty seed from each population were sown into individually labelled 5 cm pots containing the growth trial potting mix and leaf samples were harvested from each germinant after 6 weeks. Leaves were snap frozen in liquid nitrogen, lyophilised for one week (Flexi-Dry MP FTS Systems USA) and stored for later extraction. Poor

emergence was again evident in some populations and a subset of 16 of these that were both representative of the taxa identified by the multivariate analysis and had a sufficient number of germinants (15) were chosen for AFLP analysis. Total genomic DNA was extracted from germinants from each population using approx. 10 mg of dried leaf tissue which was ground to a fine powder using 3 mm tungsten carbide beads in a Retsch MM300 mixer mill followed by extraction using the Qiagen 96-well DNEasy Extraction Kit (Qiagen, Melbourne) according to the manufacturer's protocol. The amplified fragment length polymorphism (AFLP) method was performed as described by Vos *et al*. (1995) with the following modifications: the AFLP templates were prepared by digesting 250 ng of genomic DNA with enzyme combinations *Eco*RI/*Mse*I for 2 h at 37°C. *Eco*RI and *Mse*I adapters were ligated to the restriction fragments and the *Eco*I-A-*MseI*-C preamplification reaction was diluted 1:30 prior to selective amplification. An initial screening of selective primers using 12 primer combinations with four nucleotides was undertaken and three primer combinations selected (*E*-ATGC/*M*-CTA, *E*-ATGC/*M*-CTC, *E*-ATGC/*M*-CTG). The *Eco*I-ANNN primer was end-labelled with g-[^{33}P]-dATP and the AFLPs were resolved on 6% denaturing polyacrylamide gels run at 50 watts using 1X Tris-Taurine-EDTA (TTE) buffer (10.8 g Trizma base; 3.6 g Taurine; 0.2 g $Na_2EDTA.2H_20$). The polyacrylamide gels were fixed in 10% glacial acetic acid (v/v)--20% methanol (v/v) for 30 min, rinsed twice with ddH_2O, dried at 65°C overnight, and exposed to BiomaxMR film (Eastman Kodak Co., Rochester, NY) for 1-3 days.

Autoradiograms were scored visually for the presence/absence of a total of 119 AFLP loci that were numbered sequentially from largest to smallest. A binary data matrix containing the presence/absence of each AFLP band was generated and monomorphic bands were excluded. Allele frequencies were calculated with AFLP-SURV Version 1.0 (Vekemans 2002) using a Bayesian approach that assumed Hardy-Weinberg (H-W) genotypic proportions with non-uniform prior distribution of alleles

frequencies (Zhivotovsky 1999) and used to estimate gene diversity (Hj, analogous with H_e), the proportion of polymorphic loci expressed as a percentage at the 5% level (PLP) as well as total gene diversity (H_T) and genetic diversity within populations (H_w). Differentiation among populations was assessed using Wright's F_{ST} following Lynch & Milligan (1994). The number of effective alleles (n_e) was also calculated using POPGENE Version 1.32 (Yeh & Boyle 1997). Estimates of the pairwise relatedness coefficients between individuals (r) were calculated using AFLP-SURV following Lynch & Milligan's Taylor expansion (Lynch & Milligan 1994) and the relatedness matrix produced was used to extract and plot the first two principal coordinates (PCO). Similar PCO analyses were conducted with the three groups as well as for Group1 and Group2 together and individually.

Results

Multivariate analysis

Principal component analysis indicated that the West Hume populations (5 and 6) were very distinct from the remaining populations (Fig. 2a). Factor loadings identified that ridged stems (0.5414), the presence of hairs (-0.5372) and leaf shape (0.5233) were important for distinguishing among the populations for PCA1, while basal (-0.4394) and apical (0.5938) branching and height (0.6015) were important for PCA2. The West Hume populations were characterised by weakly ridged stems and mid-shape, hirsute leaves. Removing these two populations resulted in those remaining being separated into three groups (Fig 2b). Twelve populations were separated by negative PCA1 and PCA2 scores (Group1) while another 11 were separated by negative PCA1 and positive PCA2 scores (Group2). The remaining eight populations (Bendigo) were separated by positive PCA1 scores. In this analysis basal stem branching (-0.4604), ridged stems (-0.4249), the presence of hairs (0.4417) and leaf shape (-0.5594) were important for PCA1 while apical branching (-0.6473) and height (-0.6716) were

important for PCA2. Plants from Group1 were tall with strong apical branching (Fig. 3d) while those from Group2 were shorter with strong basal branching (Fig 3e). Bendigo plants were shortest with little stem branching (Fig. 3f). These differences between Group1 and Group2 may reflect differences in habit with both prostrate and upright forms being noted by experienced field collectors. Analysis of the plants with n=26 only indicated that the Group1 and Group2 populations previously identified were again differentiated with basal (-0.4575) and apical (0.5586) stem branching, height (0.5166) and leaf shape (-0.4488) important for PCA1 which accounted for 49.4% of the variation, while ridged stems (-0.7018) and retention of juvenile foliage (0.6237) were important for PCA2 which accounted for a further 23.2% of the variation.

Ploidy level

Chromosome numbers were mostly determined from single plants from all populations with the exception of Nairne. Plants were diploid in 25 of the populations with 2n=26 (Fig. 3a), and tetraploid (2n=52) in the remaining nine populations (Fig. 3b). Eight of these tetraploid populations were the Bendigo group while the ninth was Pt Lincoln (Fig. 1). Root tissue of *A. acinacea* can exhibit endopolyploidy, where there is a doubling of chromosome number within a cell, so that cells with different chromosome numbers can occur in close proximity to each other (Fig. 3c). Therefore, considerable care needs to be taken when making determinations of chromosome number. When cells with 2n=26 together with cells with 2n=52 are observed in single roots it is obvious that this is a diploid plant but it is essential to make sure that when root tip cells appear to have only 2n=52 that none with 2n=26 are present. In the tetraploid plants, cells with large numbers of chromosomes (presumed to be 2n=104) were occasionally observed. It is not clear whether the extent of endopolyploidy varies between plants or accessions but in many plants only a single chromosome number was seen despite a large number of roots being screened.

As a further check on the chromosome number observations, measurements were made of guard cell length in single plants from 14 populations. In all but one sample, the tetraploid plants from Pt Lincoln, there was a clear relationship between ploidy level and guard cell length. In the six diploid populations the mean guard cell length was 11.6µm and in the seven tetraploid ones it was 15.1µm. The plant from Pt Lincoln had the smallest guard cell length, 9.9µm but was clearly a tetraploid suggesting that this may be a different species.

Fitness

Bulk seed weight varied considerably among the populations (Fig. 4a) ranging from 0.270 g at Wedderburn (P) to 0.645 g at Berrigan (4) as did emergence and survival. Emergence was poor (≤50%) in 13 of the populations (Fig. 4b) with rates varying in the remaining 22 populations from 53% to 88%. In contrast, survival rates were generally good with 29 of the populations exhibiting >80% survival and four of these being 100%. The mean number of days to emergence ranged from 20.4 days at Deni 098 (3) to 47 days at Yapeen (G). No linear relationships were evident between seed weight and any of these emergence and survival variables. Emergence was significantly lower ($F_{2,27}$ 11.86, p<0.001) in the Bendigo populations but survivorship was similar among all three groups ($F_{2,27}$ 1.20, p=0.316). The Bendigo populations also emerged significantly slower ($F_{2,27}$ 20.36, p<0.001) while Group1 seed weights were significantly lighter ($F_{2,27}$ 7.02, p=0.004).

Significant differences in growth were also evident among the populations (all p<0.001) but although plants from Ravenswood (D) had consistently lighter roots and shoots and a smaller ASINRt:Sht, no other population-specific patterns were detected. Mean plant height ranged from 28 cm at Castlemaine (R) to 52.3 cm at Moliagul (A) (Fig. 4c) while roots and shoots were smaller at Ravenswood (D, 0.462 g and 1.248 g

respectively). Roots were heaviest at Back Boga (Y, 1.545 g) while Pt Lincoln (8) had the heaviest shoots (3.318 g). The ASINRt:Sht ranged from 0.328 at Bromley to 0.661 at Deni Forest (Fig. 4d). A significant negative linear relationship (p=0.009, R^2=20.03%) was evident between plant height and seed weight with heavier seed producing shorter plants.

There were also significant differences (all p<0.001) among the three groups with the Bendigo populations being significantly shorter with lighter roots and shoots and a lower ASINRt:Sht than the other two groups. Group1 plants were the tallest but Group2 had the heaviest roots and shoots and the highest ASINRt:Sht. Linear regressions within each group identified that for Group1 seed weight had some influence on plant height (p=0.111, R^2=25.7%), shoot weight (p=0.103, R^2=26.8%) and ASINRt:Sht (p=0.122, R^2=24.5%) but no relationships were evident for the other groups. None of the growth variables were significantly different among populations for either Group2 or Bendigo but all variables differed significantly among the Group1 populations. For this group plants at Moliagul (A) were significantly taller ($F_{10,55}$ 2.10, p=0.040) than at Lianelly (B), Kotta (T) and Deni 098 (3) while shoots weighed significantly less at Medlyns (J) ($F_{10,55}$ 3.07, p=0.004) than those from Gunbower (U). Roots also weighed significantly less ($F_{10,55}$ 3.13, p=0.003) at Medlyns (J) than those from Deni Forest (3) while the ASINRt:Sht was significantly smaller ($F_{10,55}$ 2.56, p=0.013) at Lianelly (B) than at Deni 098 (3).

AFLP analysis

The three primer combinations generated 119 scorable bands, 86% of which were polymorphic. Genetic diversity varied among the 16 populations (Table 1) with Pt Lincoln being the lowest overall. Some populations such as Deni 098 and Berrigan exhibited lower heterozygosity and PLP while for others, such as Berrimal, Yeungroon, Axe Creek, Castlemaine, the number of effective alleles, PLP and heterozygosity were

considerably higher. Comparison of means among the three groups indicate that diversity measures for Bendigo were higher than either of Group1 or Group2 which probably reflects the tetraploid status of these plants. The seed production area at West Hume (WHume95) appears to have captured a higher level of genetic diversity than the wild collection nearby (WHume99). The mean number of bands also varied within and among the populations with Pt Lincoln having the fewest (35.1) while the Bendigo tetraploid had the most (49.1). Population differentiation indicated that most of the total genetic diversity (H_t, 0.196) was apportioned within populations (H_s, 0.151) with significant subdivision among populations (F_{ST}, 0.231, p<0.001). The PCO based on relatedness identified that the West Hume sites (5 and 6) and Pt Lincoln (8) occupied different two dimensional space to the remaining populations (Fig. 6a). When these three populations were removed, some differentiation among the remaining three groups was evident with the Bendigo individuals occupying mostly negative PCO1 and positive PCO2 space while Group1 occupied positive PCO2 space and Group2 occupied mostly in positive PCO1 space (Fig. 6b).

Analysis including only Group1 and Group2 showed that most of the total genetic diversity (H_t, 0.154) was again apportioned within populations (H_s, 0.154) with significant subdivision still apparent among populations (F_{ST}, 0.156, p<0.001). The PCO for Group1 and Group2 together produced a pattern similar to that when the Bendigo was included but some differentiation among populations was evident when each of these was analysed separately. The Group1 populations of Moliagul (A) and TottingtonA (M) were more closely associated while Medlyns (J) was more allied to Gunbower (U) and Deni 098 (3) (Fig. 6a). Differentiation among the Group2 populations was stronger with Berrigan (4) being clearly distinct to all of those remaining (Fig. 6b). Most of the Sea Lake (S), Yando (V) and Yeungroon (1) individuals formed one cluster while Urana (2) and Berrimal (H) formed another.

Discussion

This study has revealed that several groups with distinctive quantitative and genetic traits as well as patterns of emergence and growth exist within the widely distributed *A. acinacea*, highlighting our poor understanding of most key revegetation species. Little information regarding the levels of genetic diversity and population structure exists for the majority of these species, and this study has identified that an integrated approach can provide important information to improve current management practices and maximise revegetation outcomes.

An understanding of genetic structure, for example, can be fundamental when determining the range over which seed should be moved. The Bendigo tetraploid, which was morphologically distinct and had higher levels of AFLP diversity than the other groups, is also highly geographically localised suggesting that genetic and/or ecological boundaries are present. Accordingly, these populations should be maintained as an entity and seed movement restricted to within its natural geographic range. A critical concern for this group, however, may be to source seed from high quality and genetically diverse populations to not only ensure initial revegetation success through seedling fitness, but to also ensure that plantings have captured sufficient genetic variability to meet future evolutionary challenges. There were some genetic differences between the two Bendigo populations sampled suggesting that variation does exist among wild populations, and in terms of fitness there were considerable differences among all of the populations with respect to seedling emergence. Indeed, the very low germination rates (< 25%) at Walmer (C), Yapeen (G) and Bromley (I) indicate that twice as much seed would need to be collected and deployed from these populations to match germination rates from other Bendigo populations. In terms of growth, however, once seedlings have germinated, no significant differences exist among populations. Establishing whether poor emergence is a result of increased inbreeding or reflects reduced genetic diversity would greatly

benefit revegetation efforts by identifying more suitable seed sources. A further consideration for deploying Bendigo seed is whether contact between this tetraploid and diploid populations will result in hybridization creating sterile triploid offspring compromising the long-term viability of both revegetation and extant populations. This may be particularly important since the hexaploid race of *A. holosericea* has resulted from hybridisation between that species and *A. cowleana* (Moran et al. 1992). Until this possibility has been explored, it would be advisable to not use seed from both ploidy levels at the same revegetation site or to deploy Bendigo seed in close proximity with natural diploid populations.

The remaining *A. acinacea* populations were divided into two groups that appear to reflect differences in habit. Both of these groups are common and widespread throughout the study region, and anecdotally can occur within 50 m of each other yet remain morphologically distinct. Combining seed of these two groups for revegetation is not recommended since we were able to demonstrate morphological and genetic divergence between them. In addition, differences in emergence rates and growth between Group1 and Group2 indicate that Group1 plants might have a competitive advantage through higher emergence rates and larger plants. It could be possible, however, to sow seed from both groups within larger revegetation sites providing sufficient distance was maintained between them to allow for separate populations to become established. Unlike the geographically restricted Bendigo group where local adaptation may not be the primary seed sourcing issue, Group1 and Group2 populations are widely distributed and local adaptation and outbreeding depression may be important considerations for deploying seed across the region, particularly since genetic relatedness within these two groups has highlighted that population genetic structure is complex and not readily predicted by geographic distribution. For example, the Group2 Urana (2) population was more genetically allied with Berrimal (H) located over 200 km south west, than with Berrigan (4) located only

50 km away. Similarly in Group1, Meldyns (J) was more aligned with Deni 098 (3) and Gunbower (U) that are located approx. 100 km to the northeast, than with Moliagul (A) and TottingtonA (M) located within 30 km. This unusual result suggests that populations such as Berrigan, Moliagul and TottingtonA may represent localised genotypes and implies that seed from these populations will need be strategically deployed.

Ideally, the possible adaptive significance of such localised genotypes would be assessed through broad scale transplantation experiments. However, the urgency of revegetation in the Murray Darling region and the time required for longer lived species to demonstrate any fitness disadvantage makes such experiments impractical. For most key revegetation species it is likely that we will need to rely on integrated studies of current genetic structure such as this to provide a useful framework upon which to base seed sourcing decisions. An important finding for this study was that no single data set provided a complete understanding of the variability within *A. acinacea*. For example, although quantitative traits and AFLPs clearly separated the West Hume populations from all others, the ploidy level did not. For the Bendigo group, quantitative traits, ploidy level and AFLPs were all important variables while Group1 and Group2 were morphologically and genetically distinct but had the same ploidy level. Overall, these results suggest that although molecular markers can provide valuable information to help ascertain seed provenances, an integrated approach that incorporates both quantitative variation and genetic markers is more appropriate for common and widespread species, particularly when these are distributed across several climatic zones and a range of habitats. An important concern highlighted by this study is that cryptic differences in ploidy levels may exist within many species. For *A. acinacea* differences in ploidy level were not readily apparent from the quantitative traits, levels of genetic diversity or mean AFLP band number and the possibility of differences in ploidy level within species adds a further layer of complexity for land

managers. This is particularly critical for long-lived species where recruitment failure brought about by the mixing of different ploidy levels may not become apparent for many years.

Seed quality is also an important issue for *A. acinacea* with considerable variability among populations with respect to emergence and growth being evident, although there was little evidence that this related to lower genetic diversity. Levels of inbreeding may be a more critical factor for progeny fitness in *A. acinacea* since many *Acacia* species are highly outcrossed (Moran et al. 1989, Muona et al. 1991) and although there is evidence of self-incompatibility in some acacias (Kenrick et al. 1986), high levels of inbreeding have been observed in others (Butcher et al. 1998, Broadhurst & Coates 2002, Elliott et al. 2002). There is also some suggestion that high correlated paternity in small and highly isolated populations of *A. acinacea* populations is reflected by reduced seedling fitness (L. Broadhurst, unpub). For seed sourced from similar populations, not only is seed production a key limiting demographic factor, but revegetation success is compromised by poor seedling viability.

The level of morphological and genetic variability detected over relatively short geographic distances was beyond what had been expected suggesting that like several other widespread *Acacia* species (Bleakley & Matheson 1992, McGranahan et al. 1997, Broadhurst & Coates 2002, Miller et al. 2002, McDonald et al. 2003), *A. acinacea* is a taxonomically complex species. There is also good evidence that the collections from Pt Lincoln and West Hume are not *A. acinacea*. The quantitative traits, disparity between the ploidy level and guard cell size and lower genetic diversity combined with the collection locality for Pt Lincoln suggest that this is more likely to be either *A. imbricata* or *A. triquerta*, while the presence of hairs on plants from West Hume suggest that these may be the closely related *A. halliana* which has appressed hairs on new shoots (Maslin 1987). Species misidentification represents a significant concern for seed collecting agencies but, given the level of morphological and genetic

variability, the wide geographic range of *A. acinacea*, and the absence of a taxonomic treatment, it is not surprising that such errors occur. This highlights the urgency of taxonomic clarification for this and other key revegetation species in the south east of Australia since the correct deployment of germplasm for revegetation can not only improve the success of such endeavours, it may also avoid potential problems following the release of taxa from biological constraints within the home range.

The high level of adaptively significant variation detected within *A. acinacea* has highlighted several important issues regarding the sourcing and utilisation of seed from native species for broadscale revegetation projects. The populations examined here exhibited significant differentiation with respect to morphology, fitness, ploidy level and AFLPs but are currently utilised by land managers as a single species, emphasizing perhaps the most significant issue for current revegetation efforts – our limited understanding of the key species being deployed in large numbers across vast tracts of land. Since the success of revegetation depends largely upon land managers making informed decisions regarding the most appropriate use of their seed sources, it is clear that considerably more information than is currently available is required. Studies such as this that encompass a range of techniques will have considerably more power for determining the most appropriate germplasm to be deployed to not only promote the long term viability of these newly created populations, but to also ensure that these revegetation efforts will not negatively impact on local populations by disrupting locally adapted traits.

Refining current revegetation practices will significantly improve the success and cost-effectiveness of these efforts. Guidelines for the collection and deployment of *A. acinacea* seed can now be refined to maintain the three groups as separate entities, to prevent mixing of the different ploidy levels, to deploy Bendigo seed within the local geographic range only, and to target collections from high quality sites. By doing so, it should also be possible to reduce the burden of sustained seed collection from

remnant vegetation which may be critical for their long term persistence. At present little is known regarding the erosion of seed banks, or the impact that seed collection has on vertebrate and invertebrate communities, many of which are likely to be important pollinator species. It may be that we are eroding the capital that remains in remnant vegetation in an attempt to replace the very communities that these remnants now represent. Given that the seed orchard at West Hume has captured high levels of genetic diversity, the establishment of similar orchards may be crucial to meet the growing demand for seed. Revegetation success may be further enhanced, once morphological and genetic boundaries have been identified, by combining data available through remote sensing and GIS with historic distribution maps generated from herbarium records to determine whether ecological indicators exist to help land managers improve revegetation efforts through more precise habitat matching. While this study has highlighted that sourcing and deployment of seed for *A. acinacea* requires a more refined approach than is currently being undertaken, it further implies that similar decisions for other species with comparable distributions and life histories also may need to be re-evaluated.

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Figure Legends

Fig. 1. Collection sites for *Acacia acinacea* seed. * indicates population used for AFLP analysis. • indicates Bendigo tetraploid populations.

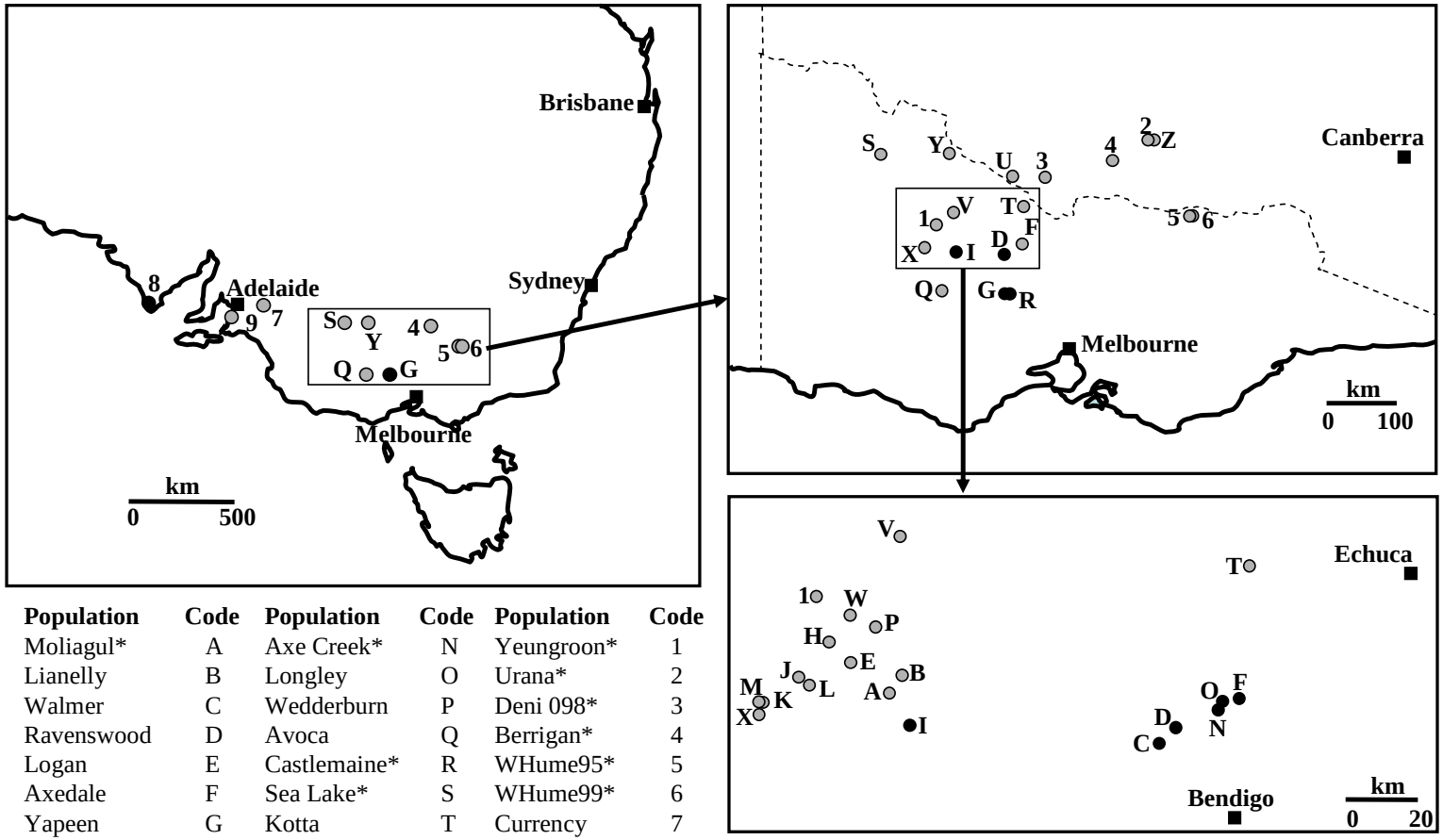
Fig. 2. Principal components analysis of six quantitative traits for all *Acacia acinacea* populations (a) and with West Hume removed (b). Population codes are in Fig. 1. Broken lines are indicative only.

Fig. 3. Mitotic metaphase in root tip cells of *Acacia acinacea* showing a diploid (a), a tetraploid cell (b) and diploid and tetraploid cells closely adjacent to each other in the same root of a diploid (c). Growth forms of the three *Acacia acinacea* groups. Group1 (d), Group2 (e) and Bendigo (f).

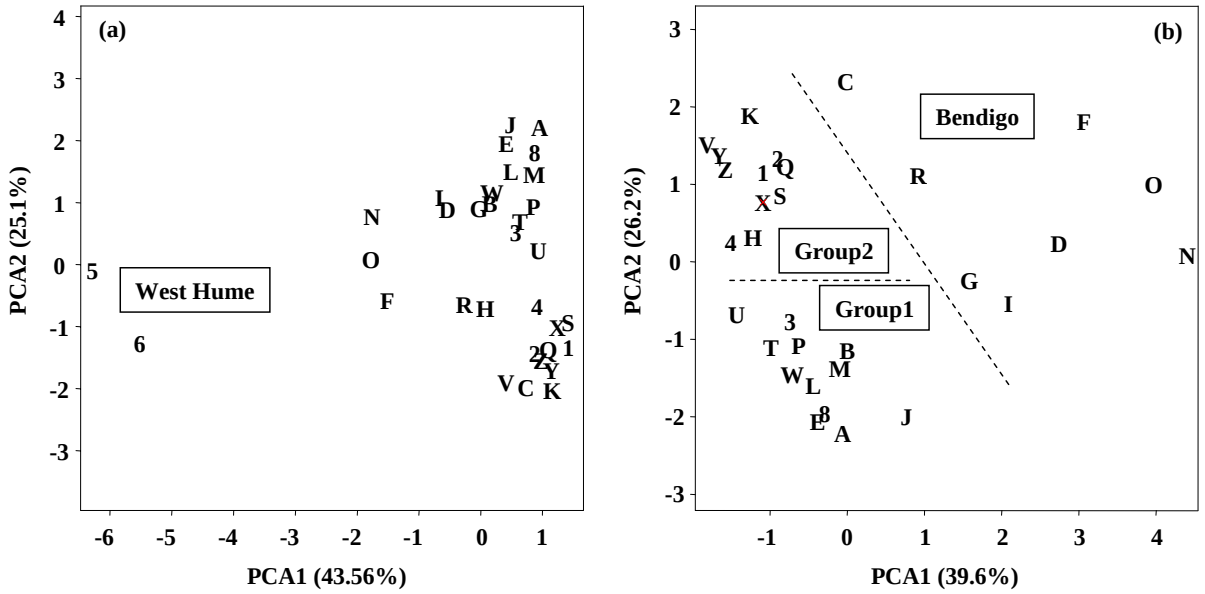
Fig 4. Mean seed weight (a), percentage emergence (b), height (c), and ASINRt:Sht (d) for each *Acacia acinacea* site.

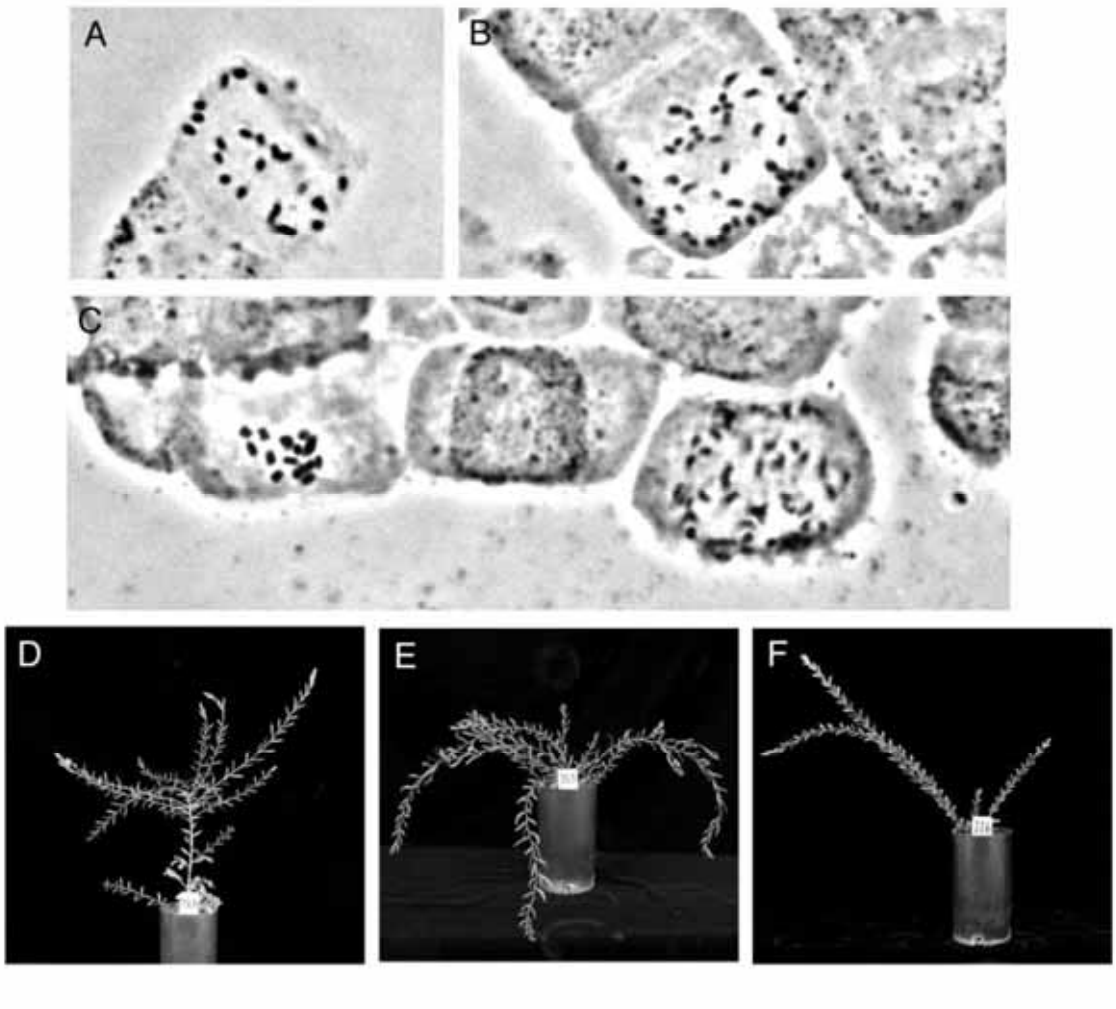
Fig 5. Principal co-ordinate representation of relatedness based on AFLP markers among all *Acacia acinacea* individuals (a), and among individuals of the three groups only (b). Numbers and letters relate to site codes in Fig. 1.

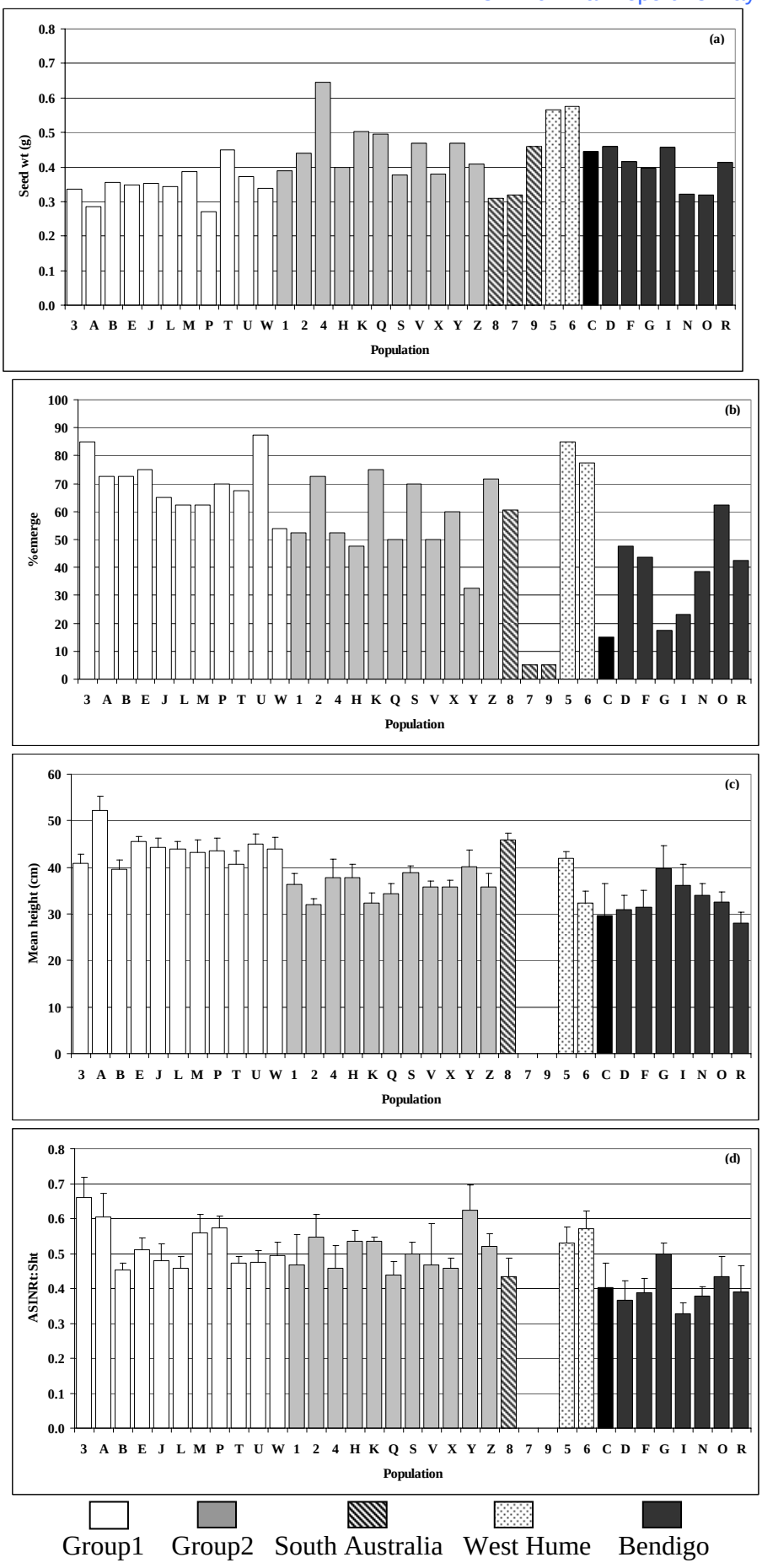
Fig 6. Principal co-ordinate representation of relatedness based on AFLP markers among Group1 individuals (a), and among Group2 individuals (b). Numbers and letters relate to population codes in Fig. 1.

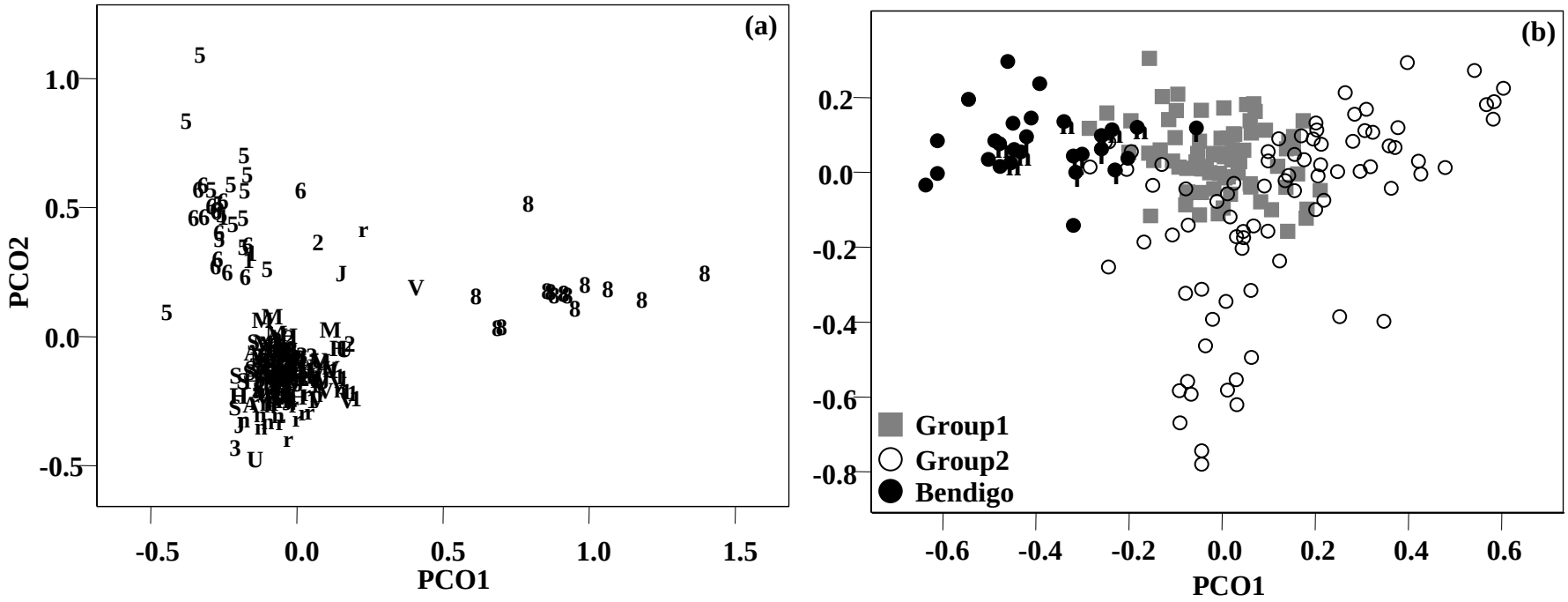


Population	Code	Population	Code	Population	Code
Moliagul*	A	Axe Creek*	N	Yeungroon*	1
Lianelly	B	Longley	O	Urana*	2
Walmer	C	Wedderburn	P	Deni 098*	3
Ravenswood	D	Avoca	Q	Berrigan*	4
Logan	E	Castlemaine*	R	WHume95*	5
Axedale	F	Sea Lake*	S	WHume99*	6
Yapeen	G	Kotta	T	Currency	7
Berrimal*	H	Gunbower*	U	Pt Lincoln*	8
Bromley	I	Yando*	V	Nairne	9
Medlyns*	J	Gowar	W		
TottingtonB	K	Rich Avon	X		
Carapooee	L	Back Boga	Y		
TottingtonA*	M	Barragunda	Z		


(a)
PCA2 (25.1%)
PCA1 (43.56%)
West Hume
(b)
PCA2 (26.2%)
PCA1 (39.6%)
Bendigo
Group2
Group1


A
B
C
D
E
F


(a)
Seed wt (g)
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
3 A B E J L M P T U W 1 2 4 H K Q S V X Y Z 8 7 9 5 6 C D F G I N O R
Population
(b)
%emerge
0 10 20 30 40 50 60 70 80 90 100
3 A B E J L M P T U W 1 2 4 H K Q S V X Y Z 8 7 9 5 6 C D F G I N O R
Population
(c)
Mean height (cm)
0 10 20 30 40 50 60
3 A B E J L M P T U W 1 2 4 H K Q S V X Y Z 8 7 9 5 6 C D F G I N O R
Population
(d)
ASINRt:Sht
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
3 A B E J L M P T U W 1 2 4 H K Q S V X Y Z 8 7 9 5 6 C D F G I N O R
Population
Group1
Group2
South Australia
West Hume
Bendigo


(a)
PCO2
PCO1
1.0
0.5
0.0
-0.5
-0.5
0.0
0.5
1.0
1.5
(b)
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
PCO1
Group1
Group2
Bendigo

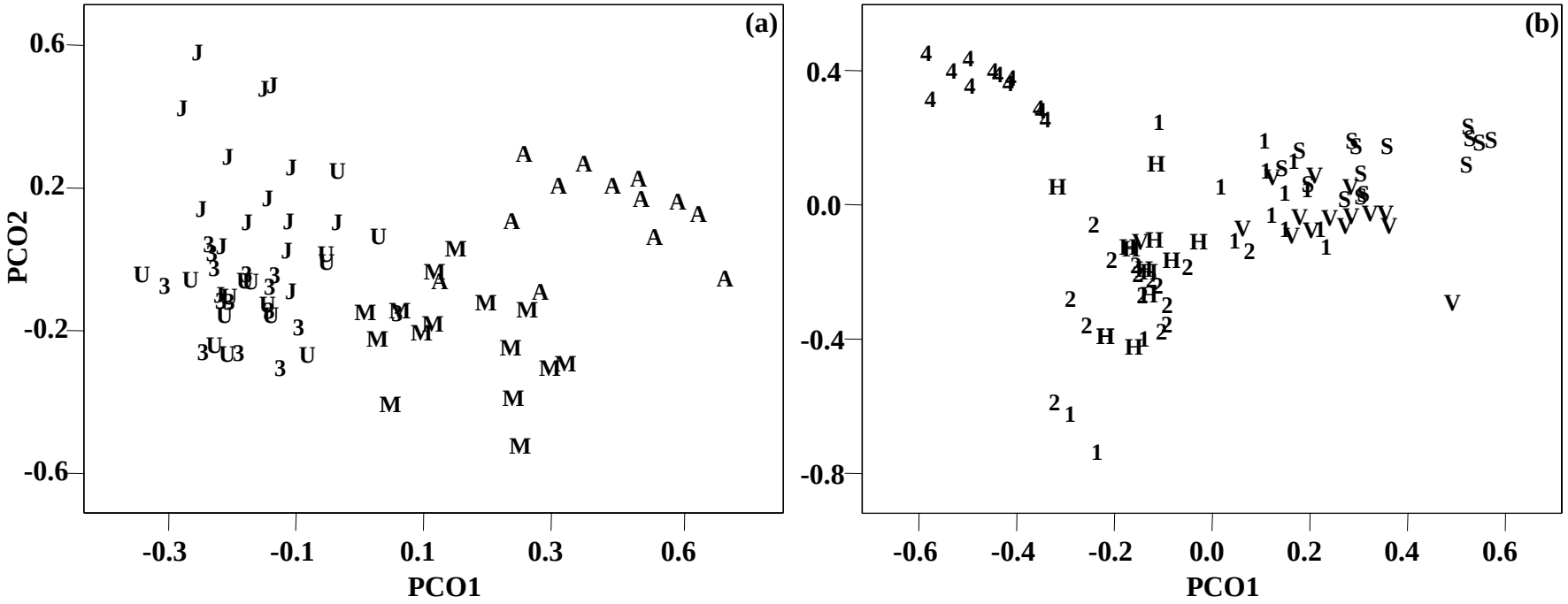

(a)
PCO2
PCO1
0.6
0.2
-0.2
-0.6
-0.3
-0.1
0.1
0.3
0.6
(b)
PCO1
0.4
0.0
-0.4
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6

Table 1. Genetic diversity measures for *Acacia acinacea* populations.

Taxon	Site	n	n_e (s.d.)	PLP	Hj (s.e.)	Mean bands
Group1	Moliagul	13	1.18 (0.03)	45.1	0.158 (0.016)	43.0
	Medlyns	14	1.20 (0.31)	45.1	0.162 (0.017)	42.1
	TottinghamA	14	1.18 (0.29)	41.2	0.142 (0.017)	38.7
	Deni 098	14	1.18 (0.32)	38.2	0.135 (0.018)	39.9
	Mean		**1.19**	**42.4**	**0.149**	**40.9**
Group2	Berrimal	12	1.23 (0.34)	46.1	0.181 (0.018)	44.7
	Sea Lake	14	1.20 (0.31)	44.1	0.156 (0.018)	40.2
	Gunbower	14	1.17 (0.29)	43.1	0.141 (0.017)	40.8
	Yando	14	1.18 (0.30)	44.1	0.147 (0.017)	42.3
	Urana	14	1.19 (0.32)	47.1	0.152 (0.017)	44.8
	Berrigan	12	1.18 (0.35)	40.2	0.138 (0.019)	45.9
	Yeungroon	15	1.24 (0.32)	54.9	0.184 (0.017)	42.1
	Mean		**1.20**	**45.7**	**0.157**	**42.9**
Bendigo	Axe Creek	14	1.23 (0.36)	50.0	0.174 (0.018)	48.8
	Castlemaine	12	1.26 (0.35)	51.0	0.201 (0.109)	49.3
	Mean		**1.24**	**50.5**	**0.188**	**49.1**
	WHume95	14	1.18 (0.31)	45.1	0.144 (0.017)	43.3
	WHume99	14	1.16 (0.31)	39.2	0.124 (0.017)	44.5
	Pt Lincoln	13	1.09 (0.22)	28.4	0.074 (0.013)	35.1

n, mean number of individuals; n_e, number of effective alleles; PLP, proportion of polymorphic loci; Hj, gene diversity.

Gene Flow and Outcrossing in a Fragmented Landscape: Implications for Seed Sourcing from Small Remnants

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ABSTRACT

The heathlands and shrublands of the Western Australian wheatbelt have been subjected to extensive recent land clearing for agriculture resulting in numerous fragmented and isolated vegetation remnants. As part of a broader study into the viability of plant populations in these remnants mating systems and patterns of gene flow were investigated in populations of the bird pollinated long-lived woody shrub *Calothamnus quadrifidis* and the insect and bird pollinated tree *Eucalyptus wandoo*. Mating system studies indicate high outcrossing rates in a number of small populations. In contrast there appears to be a trend, in both species, from low correlated paternity (probability that seeds share the same father) and lower biparental inbreeding in large populations to significantly higher correlated paternities and higher biparental inbreeding in smaller populations. Studies of gene flow patterns based on microsatellite markers indicate extensive pollen mediated gene flow into small populations from plants at least 800m away but probably only from a few trees. These preliminary findings suggest that inbreeding is likely to be a major factor affecting seed quality in small isolated populations of these species.

1.0 INTRODUCTION

Habitat fragmentation is one of the most pervasive and serious threats to the survival of plant and animal species in Australia and elsewhere in the world. The consequences of fragmentation are landscapes that consist of small isolated remnants of native vegetation in a highly disturbed or completely altered rural or urban matrix. In such landscapes many species occur as progressively small and isolated populations, with demographic and genetic stochastic processes likely to be increasingly important factors in the viability and persistence of these populations. Understanding these processes is therefore a key element to the conservation and management of remnant vegetation and the population systems that occur across those remnants.

As part of the management of remnant vegetation in fragmented landscapes there is an increasing focus on approaches and techniques for rehabilitating and restoring native plant communities. A significant issue in these landscape reconstruction initiatives is determining the best sources of seed material for re-vegetation. The issue of provenance and geographical sourcing of seed have been broadly canvassed in recent years but less has been made of other plant population attributes such as the size of the population and its level of connectivity. In terms of

population size and isolation there are a number of genetic issues that may be critical in determining the quality of the seed that might make it suitable or unsuitable for re-vegetation purposes.

The genetic consequences of fragmentation generally follow genetic theory for small isolated populations and have received significant attention over the last decade (see Young and Clarke, 2000). Two key genetic consequences of small population size are increased genetic drift and inbreeding (Ellstrand and Elam, 1993). Genetic drift and associated stochastic processes such as founder effects and bottlenecks involve random changes in allele frequencies and affect all populations regardless of size. However, in small populations (< 100 individuals) allele frequency change may be large and unpredictable (Ellstrand and Elam, 1993) and these populations will be particularly vulnerable to genetic erosion (loss of genetic variation). Increased differentiation among populations is also a likely consequence of reduced population size and genetic drift although this will depend on the level of gene flow between the affected and neighboring populations

Plants exhibit a diverse array of mating systems from predominant self-fertilization to predominant outcrossing and this will significantly influence the effect of population size on inbreeding levels. Although an association between population size and inbreeding is not always evident in plants it is probable that a substantial reduction in population size will result in increased inbreeding through increased biparental inbreeding (crossing between close relatives) or increased selfing. Biparental inbreeding is considered likely in plant populations, particularly small populations, because of often-limited gene dispersal through pollen or seed.

Both genetic drift and inbreeding can influence fitness and consequently the viability of a population and quality of seed produced through inbreeding depression and reduced fitness with increasing homozygosity. Low genetic diversity in small populations may also lead to reduced evolutionary potential limiting the population's ability to adapt to changing conditions. Inbreeding depression, in particular, has been shown to have major fitness effects on a range of traits such as seed production and germination, juvenile survival and growth, and pollen and ovule production (see Dudash and Fenster, 2000).

Another factor critical in assessing the genetic consequences of fragmentation is the effect increased population isolation will have on patterns of gene flow between populations. Gene flow is a key element in shaping gene pools and the population genetic structure of a species by maintaining genetic continuity between populations and enhancing genetic diversity within populations. For example, Prober and Brown (1994) found that small populations of *Eucalyptus albens* that were less than 250m from a larger population showed no reductions in genetic variation while more isolated remnants of similar size were genetically depauperate.

In this paper we present preliminary findings on the association between population size and mating system parameters in two species, *Calothamnus quadrifidus* and *Eucalyptus wandoo*. We also present a preliminary analysis of patterns of gene flow in two small populations of *E. wandoo*. This work was carried out as part of a broader multi-species study investigating the genetic and demographic consequences of fragmentation in plant species in the Dongolocking area of the Western Australian wheatbelt.

2.0 MATERIALS AND METHODS

2.1 Study area and species

The Dongolocking study area covers approximately 250 km^2 and consists of a highly fragmented landscape with some 82% of the land cleared for agricultural production. Although three species were investigated in the overall study, data for two species is presented here. *Calothamnus quadrifidus* is a common largely bird pollinated woody shrub with a naturally patchy distribution and is found in scrub/heath. *Eucalyptus wandoo* is a common, long-lived tree also with a patchy distribution and a key species in woodland remnants. The distribution of all populations sampled for *C. quadrifidus* is shown in Fig 1. Three *C. quadrifidus* populations F, O and B and two *E. wandoo* populations A and C were analysed for mating system variation in relation to population size (Table 1). The population sizes in *C. quadrifidus* ranged from 3128 (F) to 23 (B) and in *E wandoo* from 2315 (A) to 107 (C). Patterns of gene flow were investigated in two other populations of *E. wandoo* occurring in very small remnants, S (5 trees) and K (8 trees).

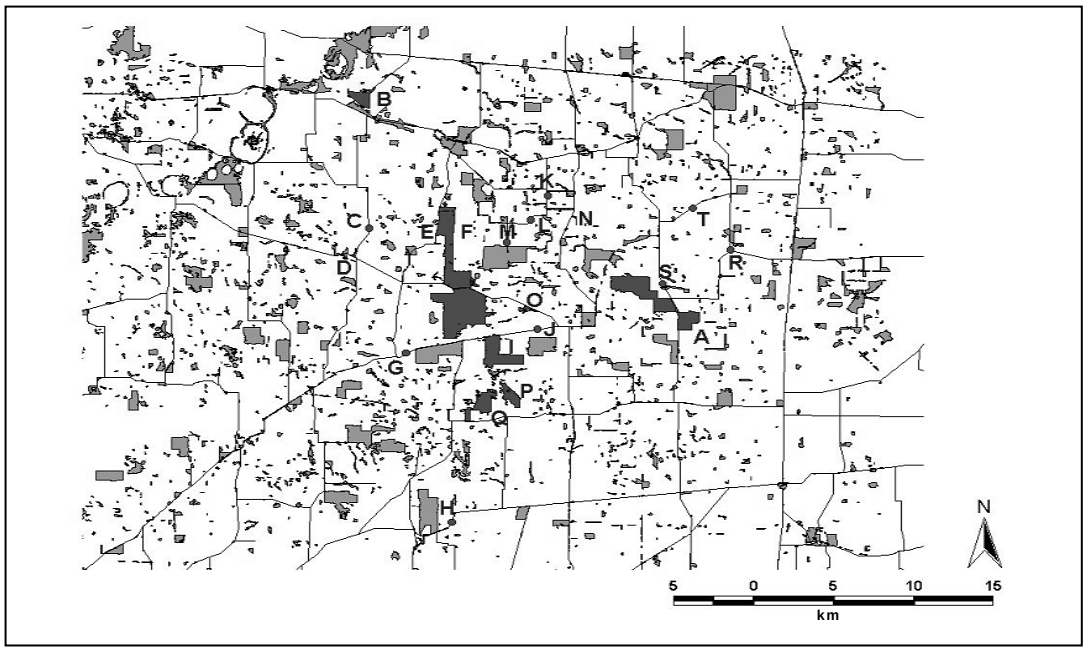

Fig 1. All populations of *Calothamnus quadrifidis* sampled as part of the Dongolocking multispecies study. Preliminary mating system studies were carried out on Populations B, F and O. Vegetation remnants are shown as shaded areas.

2.2 Mating systems

Seed material was collected from 20 plants from each population sampled for mating system studies. Up to 25 seeds per plant were germinated on moistened filter paper. Seedlings with recently emerged radicles provided the best material. Preparation of this material and the isozyme methods, using the Helena Laboratory cellulose acetate plate electrophoresis system, were described previously by Coates (1988).

Three enzyme systems were assayed in *E. wandoo* and *C. quadrifidu*s; aspartate aminotransferase (AAT,E.C., 2.6.1.1), glucose-6-phosphate isomerase (GPI, E.C. E.C. 5.3.1.9), and phosphoglucomutase (PGM, E.C. 2.7.5.1). In addition alcohol dehydrogenase (ADH, E.C.,1.1.1.1) and malate dehydrogenase (MDH, 1.1.1.37) were assayed in *E. wandoo* and malic enzyme (ME, E.C. 1.1.1.40) in *C. quadrifidus* .

Seven loci in the two populations of *E. wandoo* (aat-1, aat-2, aat-3, adh-1, gpi-2, mdh-1, and pgm-1) and six loci in the three populations of *C. quadrifidus* (aat-1, aat-2, mez-1, gpi-2, pgm-1 and pgm-2) were used to estimate mating system parameters. For both species estimates were based on 13 to 15 families for each population and 12 to 21 progeny per family.

Maximum likelihood estimates of single locus (t_s) and multilocus (t_m) outcrossing rates were based on the mixed-mating model with maternal genotypes inferred from progeny arrays (see Ritland 2002). Correlation of outcrossed paternity (r_p) was estimated according to the sibling pair model. All mating system parameters were estimated using the computer program MLTR version 2.4 (available from K. Ritland). Standard errors for the population estimates of t_s, t_m, r_p, and t_m-t_s were based on 500 bootstraps with re-sampling among maternal plants (see Ritland, 2002).

2.3 Gene flow

DNA was extracted from leaf samples from all mother trees in the two *E. wandoo* populations according to Byrne *et al*. (1993), with 0.1M sodium sulphite added to the extraction buffer and from 20 seedling germinants from each mother tree using a small scale version of the CTAB method of Doyle and Doyle (1990).

DNA samples from the mother trees and seeds were assayed for five microsatellite loci using eucalypt primers developed by Brondanni *et al*. (1998). Amplification reactions were carried out using 1.25 – 1.75 mM $MgCl_2$ in a PCR of 96C^o for 2 min; 30 cycles of 94 C^o for 30 s, 56C^o for 30 s, 72C^o for 30 s, followed by 72C^o for 5 min; and separated by electrophoresis through 8% non-denaturing polyacrylamide gels run at 300 V for 4 h, and visualised on a UV light box after staining with ethidium bromide.

The genotype of each mother tree and seedling was scored and alleles were aligned across all families. Multi-locus determination of definitively outcrossed seedlings was made directly from the raw data by identifying the presence of a non-maternal allele in the seedling. The paternity of seed on each mother tree was determined for trees together acting as pollen donors outside the population and for individual trees within the population.

3.0 RESULTS AND DISCUSSION

3.1 Mating Systems

The multilocus and single locus estimates of outcrossing rates for both *C. quadrifidus* and *E. wandoo* indicate a mixed mating system with some selfing evident in all populations of both species (Table 1). Outcrossing rates in *C. quadrifidus* are relatively high in the largest population of 3128 individuals and the smaller population of 73 individuals but are significantly reduced in the smallest population of only 23 trees. In *E. wandoo* outcrossing rates are similar for both the large and small

populations despite the large difference in population size and are comparable to outcrossing rate estimates for a range of other eucalypt species (Moran and Bell, 1983). Apart from the very small population of *C. quadrifidus* there appears to be no clear relationship between outcrossing rate and population size in either species although it is important to stress that this is a preliminary data set only and that any trend is unlikely to become evident until further populations covering a range of sizes are investigated.

Table 1. Population size and mating system estimates for three populations of *Calothamnus quadrifidus* and two populations of *Eucalyptus wandoo* as part of the Dongolocking mulitispecies study in Western Australia.

Population	No of mature plants	t_m (SE)	t_s (SE)	t_m - t_s (SE)	rp_m (SE)	Paternal Neigh-bourhood size (1/rp)
Calothamnus quadrifidus						
Population F	3128	0.87(0.05)	0.82(0.05)	0.05 (0.03)	0.05 (0.04)	20.4
Population O	74	0.89(0.05)	0.91(0.04)	-0.02(0.04)	0.10(0.02)	10.5
Population B	23	0.69(0.07)	0.63(0.07)	0.07(0.03)	0.22(0.08)	4.5
Eucalyptus wandoo						
Population A	2315	0.75 (0.04)	0.75 (0.04)	0.02 (0.02)	0.07 (0.03)	14.3
Population C	107	0.0.77(0.03)	0.66(0.03)	0.10 (0.02)	0.24 (0.06)	4.1

[a] t_m, multilocus outcrossing rate; t_s, single locus outcrossing rate; rp_m, multilocus correlation of outcrossed paternity.

In contrast to the outcrossing rate estimates there appears to be a clear trend in both species from low correlated paternity (probability that seeds share the same father) in large populations to significantly higher correlated paternities in smaller populations. This indicates that as the populations get smaller the number of fathers (paternal neighborhood size) contributing to seed production on any single plant will decline. A similar but less convincing trend is evident when comparing levels of biparental inbreeding ($t_m - t_s$), crossing between related plants. In this case, particularly in *E. wandoo,* biparental inbreeding increases with reduction in population size. Both trends indicate that mating in small populations is occurring between much smaller groups of plants that generally consist of related individuals. This trend has also been observed in smaller populations of rare plants and may not only be attributed to mating within smaller groups of plants but may also be exacerbated by pollinator behaviour. For example, in two rare woody bird pollinated shrubs, *Lambertia orbifolia* (Coates and Hamley, 1999) and *Grevillea iaspicula* (Hoebee and Young, 2001) high levels of correlated paternity were partly attributed to restricted pollinator movements among a few plants.

Increased correlated paternity and crossing between relatives in small populations of these species has implications for the potential viability of the populations over subsequent generations and their suitability as sources of seed for restoration and

re-vegetation. Elevated levels of inbreeding are likely outcomes and if associated with greater inbreeding depression may directly impact upon the fitness of the population affecting traits such as seed production and viability (see Oostemeijer, 2000).

3.2 Gene Flow

The preliminary analysis of gene flow and paternity in the two smallest populations of *E. wandoo* indicate that despite the level of isolation of the remnants and distance to the nearest tree, significant gene flow is occurring between *E. wandoo* trees across this landscape. The estimated percentage of seed produced in each tree through pollen sourced from outside the population ranged from 10% to 50% in population S (Fig 2) and 25% to 90% in population K (Fig 3). These levels of gene flow into the populations from outside sources are occurring over at least 800m.
At present there is no detailed information on pollinators of *E. wandoo* from this study although initial observations, as part of the broader Dongolocking study, indicate that both insects and birds are likely to be important. The findings here indicate that either may have the capacity to effectively carry pollen across the landscape over these distances.

Previous investigations of gene flow in eucalypts, reviewed by Potts *et al*. (2003), suggest that the minimum distance for pollen dispersal of 800m for *E. wandoo* is within the expected range for eucalypt species. Their review found that long-distance pollen dispersal may occur over distances up to 6km although generally pollen dispersal is less than 1km (Potts *et al*. 2003). Currently there is only limited empirical data on levels of pollen mediated gene flow in the Western Australian flora and in particular in the fragmented landscapes of the south west region. To date two studies on Western Australian wheatbelt eucalypt species, *E. rhodantha* (Sampson *et al.*, 1989) and *E. kochii* (M. Byrne, unpubl. data) indicate that pollen mediated gene flow may occur up to 170m and 500m respectively. This suggests that these preliminary findings for *E. wandoo* may not be unusual for eucalypt species and possibly other species with similar pollination syndromes in this region.

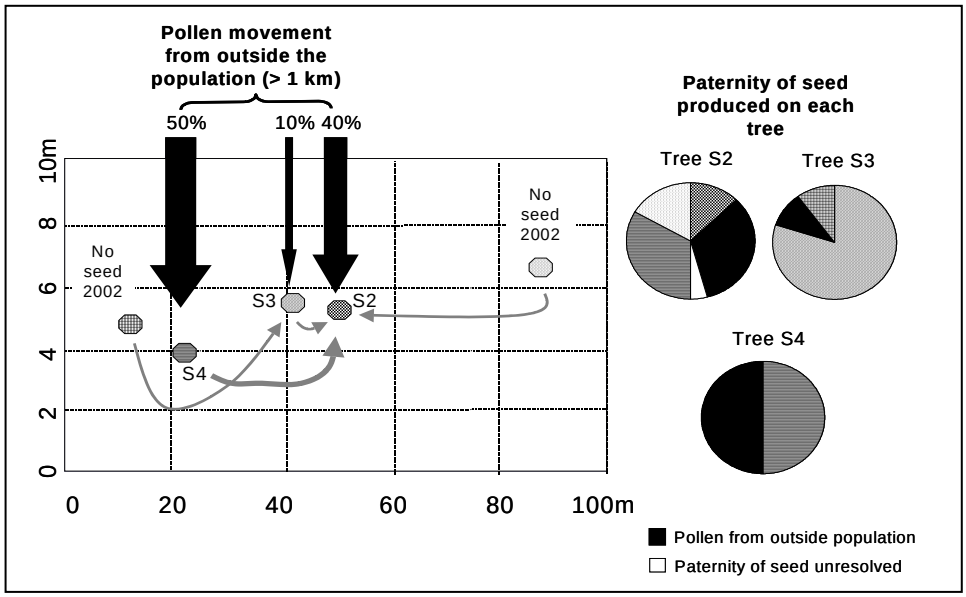

Fig 2. Patterns of gene flow in population S of *Eucalyptus wandoo.* The proportion of seed produced from different pollen sources, for each mother tree, is shown in the pie charts

The mating system studies show that as the populations get smaller the number of fathers contributing to seed production on any single plant will be substantially lower. This suggests that, despite the relatively high levels of gene flow into populations from outside sources, the number of fathers contributing to seed production on individual trees is likely to be substantially lower in these small populations than in larger populations.

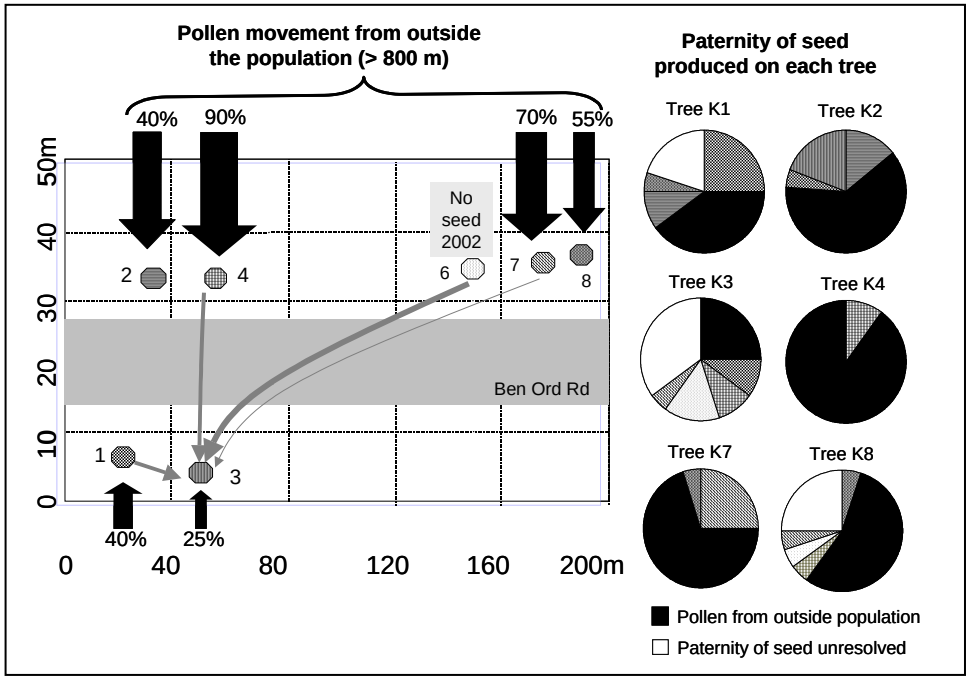

Fig 3. Patterns of gene flow in population K of *Eucalyptus wandoo*. The proportion of seed produced from different pollen sources, for each mother tree, is shown in the pie charts

In summary the preliminary findings from this study highlight important issues in the sourcing of seed from populations in remnant vegetation. The potential for elevated levels of inbreeding in small (< 100 individuals) isolated populations is high and this is likely to have a significant impact on the quality of the seed available for re-vegetation programs and the potential success of such programs. Sourcing seed from the largest population possible is the most desirable approach although this needs to be considered in the context of any broad constraints concerning local adaptation in relation to the geographical location of the source population and the target site for re-vegetation. It seems likely that at least in some instances seed sourced from a larger population may be more critical for long term re-vegetation success than seed sourced from small populations based on strict local provenance collections.

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Isolation and characterisation of microsatellites in the woody shrub, *Calothamnus quadrifidus* (Myrtaceae)

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Abstract

Microsatellite loci were developed for genetic analysis of the bird pollinated woody shrub *Calothamnus quadrifidus*. A genomic library was constructed and screened with dinucleotide and trinucleotide repeat sequences. Ten di-nucleotide microsatellite markers were developed, and polymorphism in a population of *C. quadrifidus* was investigated for six of these markers, which showed an average of 12.7 alleles per locus. Mendelian inheritance of the loci was confirmed through analysis of open pollinated progeny arrays of 10 plants. These loci will be used to study gene flow patterns between isolated populations of this species in south-west Western Australia.

Key words: microsatellites, primers, genomic library, *Calothamnus*

Calothamnus quadrifidus R.Br. is a bird pollinated woody shrub of the Myrtaceae family endemic to the south-west of Western Australia. The species is widely distributed and normally occurs in relatively large populations in sandy soil in heathland. Clearing of land has lead to increased isolation and fragmentation of the populations in the agricultural region. The ecological and genetic consequences of fragmentation are being investigated in a range of species with different life history characteristics to inform conservation management of remnant native vegetation in the area. *Calothamnus quadrifidus* has been selected as representative of a mass flowering, bird pollinated woody shrub. Microsatellite markers have been developed to investigate the effects of fragmentation on pollen dispersal and mating system in *C. quadrifidus.*

Extraction of genomic DNA from leaf material of *C. quadrifidus* was carried out as in Byrne *et al*. (1998). A genomic library was constructed using standard methodology (Sambrook et al. 1989). Genomic DNA was digested with *Sau*3*A* and ligated to pUC18 vector that had been prepared by digestion with *BamH*I and dephosphorylation with Shrimp Alkaline Phosphatase, then transformed into competent *Escherischia coli* DH5α cells (Invitrogen). The resultant 4000 colonies were screened by colony lift hybridisation onto Hybond-N+ nylon membranes using three di-nucleotide probes, AC_{13}, AG_{12}, and TA_{13}, and three tri-nucleotide probes, ATG_8, AAC_8, and GAA_8. The probes were end labelled with $\gamma 32^{P}$–dATP (Perkin Elmer Life Sciences) and hybridisation was carried out with equal concentration of the three dinucleotide 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. The membranes were stripped then hybridisation was repeated with the three trinucleotide probes. To confirm identification of clones positive colonies were picked out and grided onto new membranes then re-screened by hybridisation with the probes. Positive colonies from

the second hybridisation were picked and grown in LB media overnight at 37^{o}C. Plasmid preparations were carried out with Ultra Clean Miniplasmid Prep Kit (MoBio laboratories Inc) following the manufacturers instructions, and inserts were sequenced. Of the 74 hybridisation-positive clones sequenced, 42 (57%) contained a microsatellite sequence longer than 10 repeats. Sequences flanking the microsatellite repeat motif were analysed for 30 of these clones and compatible primer sequences were identified using PRIMER 3 software (Rozen and Skaletsky, 2000), and tested for amplification quality with AMPLIFY 1.2 (Engels, 1993). Primers could be designed for 23 (77%) of the 30 sequences, and from these, 12 primer pairs were synthesised.

Polymerase chain reaction (PCR) amplifications were carried out in a 15μL reaction volume with 20ng of *C. quadrifidus* DNA. Microsatellite loci were amplified in a reaction mix containing 50mM KCl, 20mM Tris HCl pH 8.4, 0.2 mM dNTP's, 0.3μM of each primer, 0.5 units of *Taq* DNA polymerase (Invitrogen) and variable $MgCl_2$ concentration depending on the primer as detailed in Table 1. The optimal temperature conditions for reactions for each primer pair consisted of one of two PCR programmes: (1) 96°C for 2 min: 20 cycles of 30 s at 94°C, 30 s at 69.5°C (T_a, Table 1) with a step down of 0.5°C per cycle, 30 s at 72°C; 10 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C; 2 min at 72°C; (2) 96°C for 2 min; 29 cycles of 30 s at 95°C, 30 s (except CQ 7.5 and CQ 6.17 which had an annealing time of 1 min) at 56°C (T_a, Table 1), 30 s at 72°C; 5 min at 72°C. The amplification products were subjected to electrophoresis on 8% polyacrylamide gels and stained with ethidium bromide. Of the twelve primers trialled, ten produced amplified products that could be interpreted as single loci in initial tests on six individuals from different populations (Table 1).

Variation at six of the loci was investigated further through genotyping 22 individuals of a population of the study species and genetic diversity parameters obtained by analysis of allelic data using CERVUS (Marshall *et al*. 1998) (Table 2). The level of polymorphism detected in the population and the Polymorphic Information Content of the loci were high for all loci except one, CQ2.12, that had a low number of alleles. Mendelian inheritance of the six loci was tested by analysis of segregation of maternal alleles in open pollinated progeny arrays from 10 mother plants. All loci showed segregation of maternal alleles with no evidence of null alleles as all progeny inherited a maternal allele. No pairs of loci showed linkage disequilibrium as estimated using Fishers exact test in GENEPOP (Raymond & Rousset 1995). One locus, CQ 1.10, showed significant departure from Hardy Weinberg equilibrium as estimated by Fishers exact tests in GENEPOP but was not significant when estimated using a Likelihood Ratio test implemented in POPGENE (Yeh *et al.* 1997). This is likely to be due to two alleles with high frequency rather than the presence of null alleles as the progeny array tests for Mendelian inheritance included four plants with homozygous genotypes at this locus and no evidence of null alleles was observed in the progeny. These microsatellite markers are currently being used to assess patterns of gene flow between populations of *C. quadrifidus* that have been fragmented through land clearing for agriculture in the south-west of Western Australia.

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Table 1. Details of primer sequence and optimised amplification conditions for 10 microsatellite loci identified from *Calothamnus quadrifidus*.

Locus	Primer sequences (5' - 3')	Repeat motif	T_a (ºC)	$MgCl_2$ (mM)	Size Range (bp)
CQ 1.7	**F:** CCGCAGTATCACTCCTTTATCC **R:** CTCCCCAAACCTGCCTATTC	$(GA)_{40}$	69.5	1.5	86 - 146
CQ 1.10	**F:** TGCCCACATACTTCCAGAAC **R:** CTAAACCGTCCCAAGACTCC	$(GA)_{38}$	69.5	1.5	90 - 140
CQ 2.12	**F:** ACCCCTCCGTCTTCCTAAAC **R:** GATTCTAGTCGATCAGGTCAGC	$(GA)_{36}$	56	1.0	109 - 127
CQ 5.11	**F:** CGCACAACAGAGGTCAGAAG **R:** TCCATAGCATCCAGGAAACCC	$(GA)_{35}$	69.5	2.0	94 - 171
CQ 6.1	**F:** GCGTCAACGCTTCACTTTAC **R:** ATTTGTTGAAGGCGACGAAC	$(TC)_{31}$	69.5	1.0	96 - 138
CQ 6.7	**F:** CAAGACTTGGCCTTTTGCTC **R:** AACACGACCTGCAAAACCAG	$(TC)_{33}$	69.5	1.5	96 – 147
CQ 6.17	**F:** TGTCGTCGTTGTTGTTGTTG **R:** GGGAAGTGCATTCTTTAACCTG	$(GA)_{39}$	56	1.5	158 - 160
CQ 7.5	**F:** CCTTTGTGTGTCTGTCTCTTAGACC	$(TC)_{30}ACAGA(TA)_{27}$	56	1.5	120 - 171

	R: TTTTAGCTCAGGCGTTTTGC				
CQ 4.1	**F:** TTTGCCTCTAACGCGTGTC **R:** TTTGGGCTGTTAATGAAGGAG	$(TC)_{46}$	56	2.0	107 - 135
CQ 4.3	**F:** GCTGAGTTCAGGGGAGCTATG **R:** CCGATTTTCGTTTCTTCAGG	$(GA)_{55}$	69.5	2.0	114 - 160

Table 2. Diversity characteristics of microsatellite loci for analysis of 22 individuals from a population of *Calothamnus quadfrifidus*. A, number of alleles; H_o, observed heterozygosity, H_e, expected heterozygosity; PIC, Polymorphic Information Content.

Locus	A	H_o	H_e	**PIC**
CQ 1.7	18	0.818	0.911	0.883
CQ 1.10	11	0.545	0.857	0.819
CQ 2.12	4	0.364	0.518	0.418
CQ 5.11	16	0.727	0.939	0.912
CQ 6.1	12	0.864	0.905	0.873
CQ 6.7	16	0.727	0.884	0.853
Mean	12.8	0.664	0.836	0.793

Non-provenance genetic issues for native seed sourcing

ACMER workshop paper

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Abstract
As revegetation targets become more ambitious, and demand for native seed continues to outstrip supply, increasing the efficiency with which native seed are sourced and deployed to maximize replanting success is an important element to achieving land management objectives. From a genetic prespective, maximizing local adaptation by sourcing seed locally (the precautionary principle) is intuitively appealing and has been widely adopted and formalized within collecting guidelines. Here we discuss the other elements of genetic quality of seed crops that can directly affect their fitness. These include: 1) genetic diversity; 2) degree of inbreeding and; 3) genetic integrity (or degree of hybridization). The underpinning genetic theory is discussed and illustrated with data from Australia case studies where possible. Based on these results the argument is made that these dimensions of genetic healthy can be as equally important as local adaptation in determining the utility of a seed crop for revegetation. Moving forward with a more utilitarian and inclusive concept of genetic quality, and away from the dominance of local adaptation and genetic provenance as the overriding genetic seed sourcing issues, is a critical step in attaining the seed use efficiencies that are so crucial to the success of large-scale revegetation in Australia. Explicitly quantifying the relative importance of these different components of "genetic health", and their influence on genotype fitness, in different landscapes, and for different suites of species, is a fundamental first step to achieving this important paradigm shift.

Introduction

The past ten years has seen a massive increase in native revegetation taking place in most Australian states as national, state and regional agencies attempt to manage the ongoing threats to both natural and production landscapes presented by dryland salinity and soil acidification.

Both the scale and the ecological complexity of the plantings has increased, with a broader array of native species being reintroduced into agricultural areas with three broad goals: 1) extension of current high quality remnant vegetation; 2) rehabilitation of degraded vegetation and; 3) revegetation of agricultural land. Some implementation challenges are unique to each of these different objectives. For instance reestablishment of healthy soil microfloras can be a critical part of revegetation *de novo* (objective 3), while plantings aimed at rehabilitation (objective 2) may rely on the remnant soil flora. Similarly, a working knowledge of succession, symbioses and community assembly rules is also critical for reclamation of agricultural land (objective 3) , whereas a basic level of residual functionality can be assumed when extending current remnant vegetation (objective 1). One issue that is common to all three major types of revegetation activities however is the availability of high quality seed of a broad range of native species.

The amount native seed required for revegetation activities has grown significantly over the last ten years to the point where tonnes of seed are now being sourced annually (much of it from remnant vegetation). Despite some upturn in supply, seed availability is still far outstripped by demand and, in the absence of development of large-scale seed production areas, this is likely to continue into the future. Constraints may also have to be placed on some current seed sourcing practices as the ecological implications of removing large quantities of seed from remnant vegetation become apparent. Such effects may be impacts both on the short-term ecological viability of source populations themselves, as well as negative effects on the survivorship and demographic viability of seed eating animal populations.

Given that simply increasing supply is unlikely to meet ongoing increases in seed demand using current revegetation practices, at least in the medium term, the other option is to use the seed that is available more efficiently. A key issue here is to maximize the genetic quality of the seed used for revegetation as this will provide significant efficiencies through improved germination, establishment, growth and subsequent reproduction.

There are several relevant elements to genetic quality in this context. The first of these, which is generally recognized, is the concept of local adaptation, that is that particular genotypes within a species are adapted to their local environment and so use of local genetic material is likely to produce the best result in terms of revegetation success. This is the genetic management issue that has been most readily appreciated by agencies and groups involved in revegetation. It is usually labeled the “genetic provenance” issue and is widely recognized through formalization of the requirement to use local provenance seed whenever possible to maximize local adaptation and so promote replanting success. This practice is called the “precautionary approach” and now has a significant influence in the way native seed are sourced for revegetation, with many sets of guidelines advocating use of local genetic material for most plantings e.g. ANPC (1997a & b) and Florabank Guideline 5 (Mortlock & ATSC 1999). In the absence of much information regarding the scale of adaptively significant genetic variation however, determination of what constitutes locally adapted gene pools is difficult and, in practice, as local as possible is often the reality of the situation.

While use of locally adapted genetic material seems intuitively like a good idea, it must be remembered that local adaptation is only one dimension of genetic quality. Other genetic issues may also influence the fitness of seed crops. These include the level of genetic diversity, the amount of inbreeding and the genetic integrity of the seed crop. These genetic issues are rarely if ever considered in seed souring and yet are particularly relevant to many situations where seed are sourced from local remnant vegetation stands which are often themselves small and degraded. As these are often preferred sites for seed sourcing because they are the only local populations left, and they are easy to access, understanding the influence of these factors on remnant seed crop quality, and the implications for revegetation success, is equally important as managing to promote local adaptation. Indeed it is useful to explicitly consider the idea that there may be tradeoffs between local adaptation and genetic quality in terms of these other factors when making decisions as what seed sources will maximize revegetation success. It is these “non-provenance” genetic seed quality issues: 1) genetic variation 2) inbreeding and; 3) genetic integrity or hybridisation, that are the focus of this paper. For each the genetic theory is briefly reviewed and, where possible, an illustrative case study presented.

Genetic diversity

Low genetic diversity in a seed crop can directly limit the viability of revegetation through effects on fitness, mate availability and reduced evolutionary potential. Loss of variation is generally associated with low population size, which is often the case in the remnant stands from which seed are commonly sourced, and results from a combination of initial sampling effects or founder events and subsequent random genetic drift (Hartl 1988). Founder events and genetic drift affect all populations, regardless of size. However, in large populations their effects are very small and easily counteracted by the generation of new genetic variation by mutation and buffered by the influence of stablising selection. As size becomes smaller, low mutation rates cannot replace variation as rapidly as it is lost, and selection coefficients are effectively reduced, resulting in a greater influence of genetic drift. These two factors: founder effects and genetic drift; affect different types of genetic variation in different ways, primarily due to the mode of gene expression in the case of single locus vs polygenic variation, and the difference in functional constraints on gene action in the case of neutral genes vs genes of major effect.

Genetic variation at single gene loci is measured by parameters such as allelic richness (A) – the number of alleles per locus, and gene diversity (H_e), which is commonly referred to as expected heterozygosity and measures the average number of loci within an individual that are expected to be heterozygous assuming random mating within populations. In the absence of immigration, mutation and selection, the mean expected change in allele frequency (Δq) due to one generation of random genetic drift is:

$$\Delta q = q\,(1-q) / 2N_e$$

(where N_e is the variance effective population size) Falconer (1989)

The probability of fixation for a particular allele is equal to its initial frequency. Losses of gene diversity with reduced population size are expected to be less than for allelic richness as it is effected relatively little by low frequency alleles, which are the ones most likely to be lost due to genetic drift.

Available empirical data for plants confirm that reductions in single locus genetic variation accompany reductions in population size and show that this is generally a

logarithmic rather than a linear relationship (Young et al. 1999; Prober & Brown 1994; Buza et al. 2000 van Treuren et al. 1991; Raijmann et al. 1994). However, as predicted, population size has generally been found to have little effect on gene diversity.

Such losses of allelic richness in remnant populations and the resulting low diversity in their seed crops become important when it affects genes that directly effect plant fitness and population viability, for instance those conferring disease resistance. Another good example is the group of genes controlling self-incompatibility in flowering plants. Genetically controlled homomorphic self-incompatibility systems occur in a range of angiosperm plant families - with an estimated half of all flowering plants being self-incompatible (Richards 1997). These systems provide a mechanism for pollen-stigma recognition that prevents fertilisation occurring between pollen and ovules of plants that share genes at the incompatibility locus. Genetic control of the system is usually by a single gene and can be either gametophytic (haploid control) of sporophytic (diploid control). Theoretically sporophytic systems are more effective at preventing mating between relatives as only a single allele need be matched between two plants to prevent fertilisation. In practice, the occurrence of dominance relationships among alleles in sporophytic systems occurs commonly, resulting in an less efficient system than expected (Richards 1997).

In large populations, with high genetic variation, self-incompatibility systems represent effective mechanisms to limit inbreeding and avoid the negative fitness effects that come with it. However, it has been suggested that in small populations, if *S* alleles are lost and overall allelic richness becomes low, incompatibility systems may become a demographic liability as they will result in significant reproductive limitation due to there not being enough inter-compatible mating types. Indeed, for either gametophytic or sporophytic dominant systems there must be at least three alleles in a population for any mating to be possible, while for plants exhibiting sporophytic dominant systems a minimum of four alleles are required.

Two studies have shown that loss of *S* alleles is the cause of poor seed set in small populations of the endangered plants *Hymenoxys acaulis* var. *glabra* (DeMauro 1993) and *Aster furcatus* (Les *et al.* 1991). In both cases only a single population was studied. More recently extensive investigations of the self-incompatible grassland herb *Rutidosis leptorrhynchoides* in southeastern Australia, Young *et al*. (2000) have shown that populations of fewer than 200 plants exhibit 50% reductions

in the numbers of *S* alleles found in their seed crops (See Figure 1). In this case adopting the precautionary principal and sourcing seed locally from only a single small population will severely compromise the demographic viability of revegetation efforts owing to low cross-compatibility in the seed crop and subsequent low reproductive output of the replanted population.

There are two possible solutions. First, source seed from only large populations with high *S* allele diversity – however as there are few of these and they are geographically widespread so that issues of local adaptation may become a consideration. The second possible solution is to source from several small local populations and mix these collections to increase the chance of sampling different *S* alleles among local seed crops. This strategy should provide the levels of genetic variation required for populations to function reproductively, while also maintaining local adaptation.

Inbreeding and Fitness

Genetic variation at the level of the individual is measured in terms of heterozygosity (H_o). The number and frequency of alleles at a locus, the amount of inbreeding and the type and intensity of natural selection determine the level of heterozygosity within a population. Maintenance of heterozygosity in seed crops used for revegetation is important because low heterozygosity is often associated with reduced fitness (inbreeding depression).

For neutral loci, and assuming no inbreeding, heterozygosity is the same as gene diversity and is lost due to random genetic drift at a rate of:

$$\Delta H_e = -H_e / S$$

where *S* is the number of gametes (ie twice the population size for diploid species).

As populations become small however, such as in remnant vegetation, increased selfing (when possible) or mating among related individuals will also work to reduce heterozygosity such that:

$$\Delta H_e = H_e F$$

Where *F* is the average coefficient of relatedness between parents (equations follow Hartl (1988)).

Changes to the pollination system, such as in the composition of pollinator guilds, or effects of population size or density on the behaviour of individual pollinator species, can also increase levels of inbreeding e.g. Karron *et al.* (1995).

Thus in seed crops harvested for revegetation from small isolated populations in degraded remnant habitats, low heterozygosity owing to inbreeding and reduced fitness can be expected. Allozyme studies of several plant species have examined the relationship between remnant population size and heterozygosity directly and the results have demonstrated this. For example, both *Eucalyptus albens* (Prober & Brown 1994), and *Gentiana pneumonanthe* (Oostermeijer et al. 1994) show positive logarithmic relationships between population size and heterozygosity observed in seed crops.

In terms of revegetation, the main concern with reduced seed crop heterozygosity, is the association between decline in heterozygosity and negative fitness effects due to inbreeding depression and the effect this may have on population viability. There is now excellent evidence that inbreeding can have large effects on a wide range of fitness components in plants ranging from seed weight to germination and growth characteristics and reproductive output (See Fenster and Dudash 1994 for a review) all of which are important considerations when thinking about the performance of seed in revegetation. A good example of the possible effects of inbreeding affecting seed crop quality and utility for revegetation is the case of the herbaceous grassland pea *Swainsona recta.*

Swainsona recta is an endangered grassland herb persisting in 17 small populations ranging in size from 3-400 plants. The species is an autotetraploid and as such might be expected to maintain high heterozygosity and exhibit low inbreeding depression even in small populations. However, direct measurement of levels of inbreeding in *S. recta* using genetic markers show that there is a 50% reduction in heterozygosity in the seed crop of small populations due to selfing or mating among relatives (Buza et al. 2000). Growth experiments comparing inbred seeds from small populations (<10) with outbred ones from large (>100) populations suggest that this inbreeding is accompanied by strong reductions in fitness (See Figure 2).

These results indicate that sourcing seed for revegetation locally is a mistake if local *S. recta* populations are small. In this case however, unlike with *Rutidosis leptorrhynchoides*, mixing seed from several small populations will not alleviate the problem, because all seed crops from small populations are inbred. For *S. recta*, seed collections must be made from large populations, despite the fact that these can be a long way from the target revegetation site. Given this, if local adaptation is strong for this species (and there is no information available on this at present), the relative tradeoff between the negative effects of maladaptation and inbreeding depression must be considered.

Genetic integrity and hybridisation

Hybridisation, the mating between different species (usually with a genus) is common in plants, and particularly common in several large Australian plant families such as the Myrtaceae and the Fabaceae. Hybrids can be more vigorous than either of the parental species regardless of environment (heterosis), or may be maintained by being more suited to intermediate habitat (Moore 1977). However, it is more common for hybrids to be less fit than their parents over the majority of habitat conditions (Barton & Hewitt 1985). In this sense, hybridisation can be viewed as an extreme form of outbreeding depression.

Thus, if hybridization rates are elevated in disturbed remnant habitats where seed are often collected, then the inferior seed crops may negatively effect growth rates of re-established populations. This is a similar argument to that made regarding the threat of inbreeding depression. The second issue unique to hybridization is that of maintenance of the genetic integrity of the species being used in revegetation. Planting hybrids may well be viewed as acceptable if the goal of the planting within the landscape is simply ecological utility e.g. to pump water, or to alleviate erosion. However, if the aim is to redevelop native plant communities for biodiversity conservation, the deployment of hybrid genotypes is questionable. It can be argued that the genes of the target species are all still represented in the seed crop, so from one perspective there is genetic conservation. However, the multilocus gene combinations that uniquely defined species are gone. As these represent a unique evolutionary end point this can be seen as undesirable. More complex are the situations where the novel multilocus genotypes generated by hybridisation actually do impact in a negative way on the roles that the hybrids can play within a functioning ecological community. Such effects can be easily envisaged to involve complex or

symbiotic interactions, for instance if hybrids between two species with different levels of nectar production produce insufficient nectar to attract pollinators of either species.

Obviously, in terms of sourcing seed for revegetation, the key issue is whether the seed crops harvested from small or degraded remnant habitats contain large numbers of hybrids. Data on this issue are few, however it certainly seems likely that this could be the case, as natural hybridization is often associated with disturbance. In one of the few studies to explicitly compare hybridization rates between intact and fragmented populations of a plant, Field et al. (2004), found increased production of hybrids in the seed crops of small degraded *Eucalyptus aggregata* populations in southeastern Australia.

Conclusions

As demand for native plant seed for revegetation activities continues to rise and, for the foreseeable future outstrip supply, increasing the efficiency with which seed are utilized will continue to be an important element in achieving regional and national land management objectives. While the importance of using locally sourced seed to maximize environmental adaptation is well recognized, the potential importance of the genetic quality of seed crops as determined by the amount of diversity they contain, their level of inbreeding and their genetic integrity is not. The data presented above show that is quite possible that genetic limitations other than environmental adaptation can severely compromise the utility of even locally collected seed crops – especially when these are sourced from small degraded remnant populations, as is often the case. From this it becomes clear that the use of local seed at all costs, or even the broad application of the precautionary principle in seed sourcing, may well be flawed in many common situations when dealing with revegetation projects in highly fragmented and disturbed landscapes.

The idea of a tradeoff between the utility of sourcing locally to maintain adaptation, and sourcing from large (possible distant) populations to maintain diversity, outbreeding and minimise hybridisation must now be seen as fundamental to development of future seed sourcing strategies. Thus it is critical that we begin to understand the shape of the tradeoff between these dimensions of “genetic health” and to what degree they each contribute to the fitness of a seed crop planted into an environment. This knowledge is now fundamental to making efficient seed sourcing and deployment decisions. For example, Figure 3 illustrates three different response

surfaces for fitness representing different relative effects of local adaptation and, in this case, inbreeding. The first of these is where inbreeding and adaptation are equally important. The second represents the situation in which inbreeding has a greater effect on fitness than local adaptation – for instance when local populations are very small and highly inbred. The third represents local adaptation being critically important and the effects of inbreeding being relatively mild – possibly a situation where environmental variation is strong, and local seed source populations are still large and undisturbed.

Moving to this more inclusive understanding of the genetic make up of seed crops, and away from the dominance of local adaptation and genetic provenance as the overriding genetic seed sourcing issues, is a critical step in attaining the seed use efficiencies that are so crucial to the success of large-scale revegetation in Australia. Beginning to quantify the relative importance of these different components of "genetic health": 1) local adaptation; 2) genetic diversity; 3) inbreeding and; 4) genetic integrity, and how these tradeoff to determine genotype fitness in different landscapes, and for different suites of species that have varied ecologies, is a fundamental first step to achieving this important paradigm shift. As such, this represents one of the most a pressing research issue for revegetation ecology and genetics.

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Figure 1. Loss of *S* alleles (solid symbols) and allozyme alleles (open symbols) with reduced population size in fragmented populations of the grassland herb *Rutidosis leptorrhynchoides* (reproduced from Young et al. 2000)

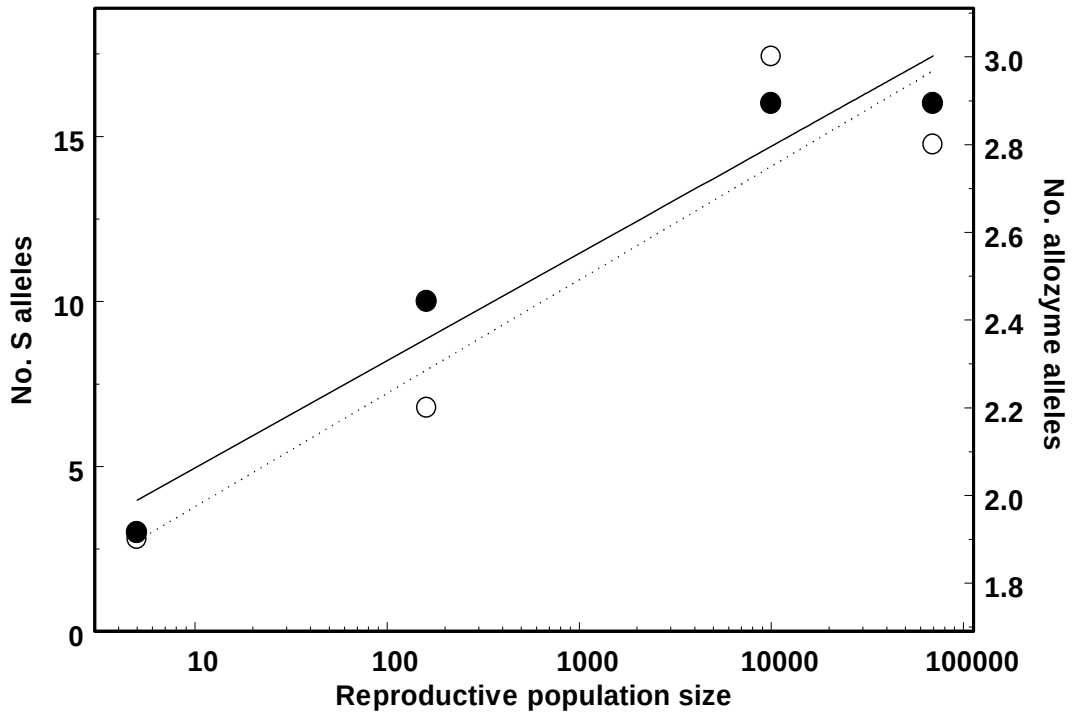


Figure 2. Increased inbreeding and reduced fitness with reduced population size in fragmented populations of the grassland herb *Swainsona recta* (reproduced from Buza et al. 2000)

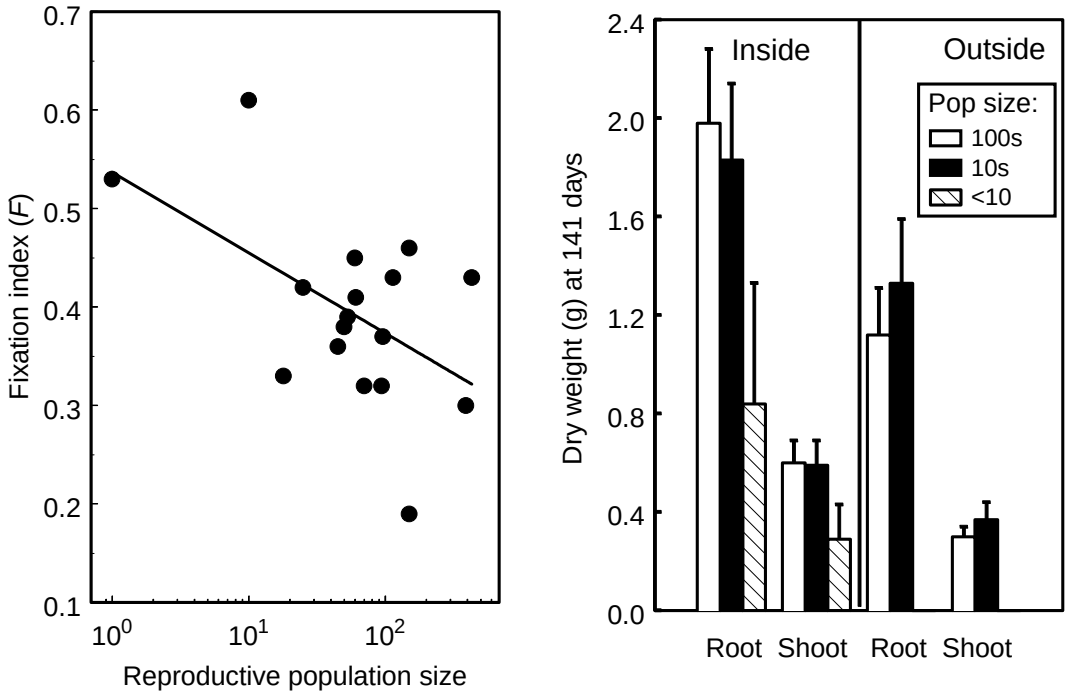
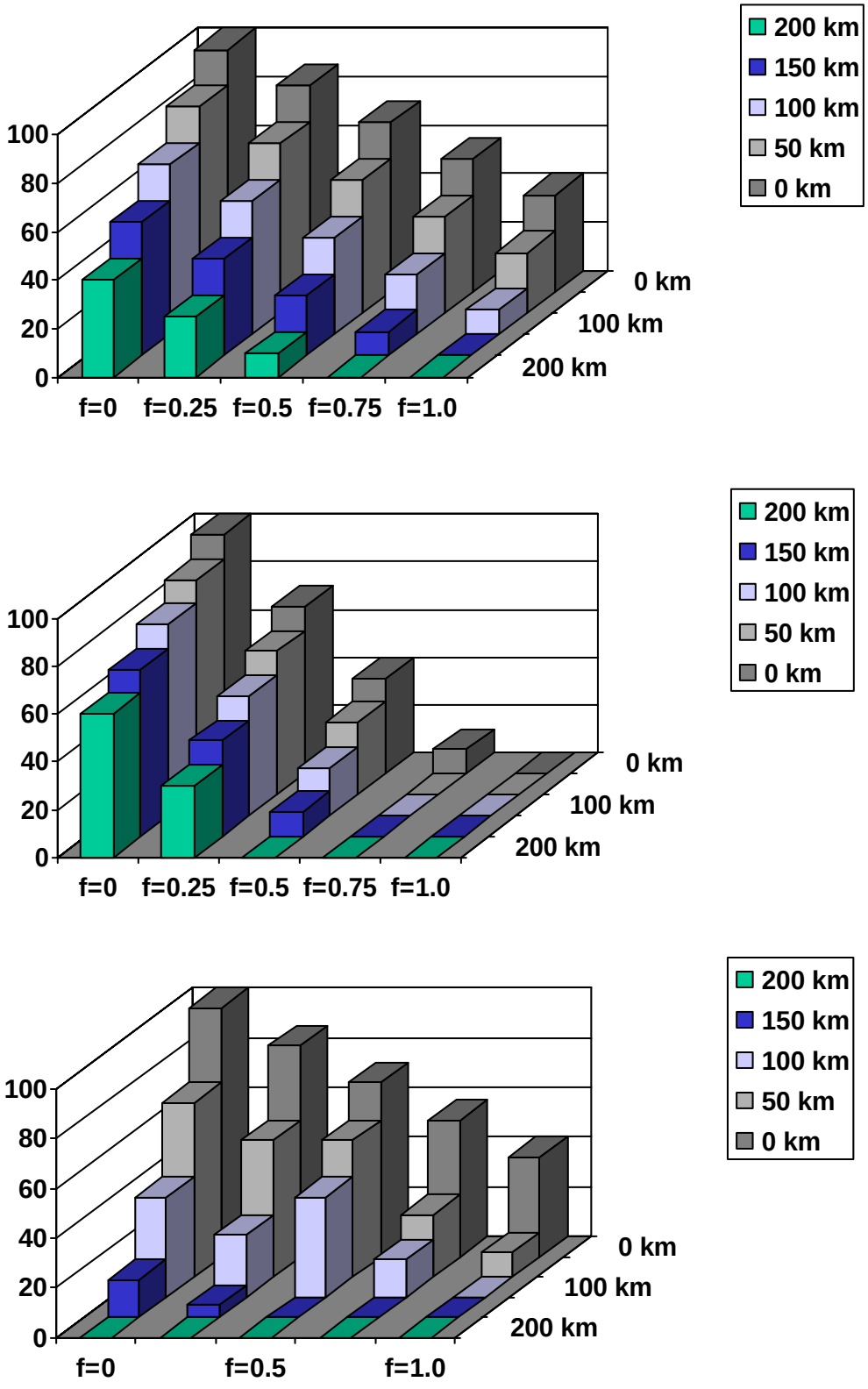


Figure 3. Tradeoff between local adaptation and inbreeding in determining the relative fitness of seed crops when: a) effects of inbreeding and local adaptation are of similar magnitude; b) inbreeding effects are stronger than local adaptation and; c) local adaptation is stronger than inbreeding



APPENDIX 7.5: Communications Achievements

Publications

Broadhurst L.M., Young A.G., Thrall P.H. and Murray B.G. (in press) Sourcing seed for *Acacia acinacea*, a key revegetation species in south eastern Australia. *Conservation Genetics*.

Coates D. J., Byrne M., Elliott C., Sampson J. and Yates C. (in press) Gene flow and outcrossing in a fragmented landscape: implications for seed sourcing from small remnants. In *Proceedings of the Fifth Australian Workshop on Native Seed Biology*. Eds Adkins, S., Bellairs, S. and Coates D. Australian Centre for Minesite Rehabilitation. Brisbane.

Elliott, C and Byrne, M. (in press) Isolation and characterisation of microsatellites in the woody shrub, Calothamnus quadrifidus (Myrtaceae). *Molecular Ecology*.

Young A.G. (in press) "Non-provenance genetic issues for native seed sourcing". In *Proceedings of the Fifth Australian Workshop on Native Seed Biology*. Eds Adkins, S., Bellairs, S. and Coates D. Australian Centre for Minesite Rehabilitation. Brisbane.

Colin J. Yates, David J. Coates, Carole Elliott And Margaret Byrne (submitted) Bird pollination and mating system variation for a shrub in fragments of species rich kwongan in south-west Western Australia. *Biodiversity and Conservation.*

Publications in preparation

Broadhurst L.M., Young A.G. and Gregory E. Demographic constraints to population persistence in fragmented *Acacia dealbata* populations from south east Australia.

Broadhurst L.M., Young A.G. and Gregory E. Genetic diversity and mating system parameters influencing fragmented *Acacia dealbata* populations from south east Australia.

Broadhurst L.M., Young A.G. and Gregory E. Cryptic genetic diversity in the south east Australian grassland herb *Swainsona sericea*.

Broadhurst L.M., Young A.G. and Gregory E. Demography and fitness in the south east Australian grassland herb *Swainsona sericea*.

Byrne M., Elliott CP., Yates C. and Coates DJ. Pollen dispersal in Eucalyptus wandoo, a dominant tree in a fragmented landscape in Western Australia.

Byrne M., Elliott CP., Yates C. and Coates DJ. Pollen dispersal in Calothamnus quadrifidus, a bird pollinated shrub in a fragmented landscape in Western Australia.

Byrne M., Elliott CP., Yates C. and Coates DJ. Genetic effects of fragmentation on a dominant tree species.

Coates D.J., Gage C., Elliott C. P., Yates C.J and Byrne M. Genetic consequences of population fragmentation in a bird pollinated shrub Calothamnus quadrifidus and an insect pollinated shrub Eremaea pauciflora

Coates D.J., Yates C.J., Elliott C.P., Sampson J. and Byrne M. Pollination and mating system variation for a tree in fragments of eucalypt woodland in south-west Western Australia.

Yates C.J., Elliott C.P., Byrne M. and Coates D.J. Seed production and offspring fitness for a tree in fragments of eucalypt woodland in south-west Western Australia.

Yates C.J., Elliott C.P., Coates D.J. and Byrne M. Bird pollination and mating system variation for a shrub in fragments of species rich kwongan in south-west Western Australia.

Yates C.J., Elliott C.P., Byrne M. and Coates D.J. Seed production and offspring fitness for shrub in fragments of species rich kwongan in south-west Western Australia.

Presentations

Biodiversity Bites Seminar Series 6 November 2002

Broadhurst L. and Thrall P. “Bushland on Life Support” .

Consequences of Habitat Fragmentation Workshop Sydney 4-5 July 2003.

Broadhurst L. and Young A. ‘Size does matter for *Acacia dealbata*’.

Coates D.J., Byrne M., Gage C., Yates C. and Elliott C. “Genetic and ecological consequences of habitat fragmentation in two woody shrubs (*Calothamnus quadrifidus* and *Eremaea pauciflora*) and the tree (*Eucalyptus wandoo*) in the Western Australian wheatbelt”

Fifth Australian Workshop on Native Seed Biology Brisbane 20June 2004.

Coates D. J., Byrne M., Elliott C., Sampson J. and Yates C. “Gene flow and outcrossing in a fragmented landscape: implications for seed sourcing from small remnants”.

Young A.G. “Non-provenance genetic issues for native seed sourcing”.

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Broadhurst L. “The role of genetic diversity in revegetation efforts”.

Broadhurst L. “Landscape fragmentation and remnant viability”.

Broadhurst L. “Collecting seed to maximise genetic diversity”.

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Posters

Project outline “Piecing Together the Patches “

Consequences of Habitat Fragmentation Workshop Sydney 4-5 July 2003.

Field D., Young A., Whelan R. and Ayre D. “The role of habitat fragmentation and disturbance in promoting hybridization in *Eucalyptus aggregata*”.

Gage C., Coates D. and McComb J. “Fragmentation and the effects of population size on genetic diversity and seed set in *Eremaea pauciflora* (Myrtaceae)”.

XIX International Congress of Genetics (IGC) Melbourne 6-11 July 2003.

Broadhurst L. and Young A. “Population viability in an agricultural landscape: lessons from *Acacia dealbata*”.

Byrne M., Yates C., Elliott C. and Coates D. “Genetic and ecological consequences of population fragmentation in *Eucalyptus wandoo*”.

Coates D., Yates C., Elliott C., Byrne M. and Sampson J. “Genetic and ecological consequences of population fragmentation in the bird and mammal pollinated woody shrub *Calothamnus quadrifidus* ”.

Field D., Young A., Whelan R. and Ayre D. “The role of habitat fragmentation and disturbance in promoting hybridization in *Eucalyptus aggregata*”.

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Radio Interviews

- ABC National Regional Radio (Horsham radio) Andrew Young “Patching up the native ‘gene pool” 1 May 2002.
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- “Supporting bushland with science” Bushland on Life Support Dec 2002.
- “Out on a limb: the meaning of isolation for remnants” Thinking Bush April 2003.
- “Genetic variability of remnant plant communities” Monaro Grassland Mail Spring 2003.
- “Right on track, monthly news feature” Conservation News June 2003.
- “Conserving genetic diversity at the patch and landscape scale” Thinking Bush June 2004.

Other

- CSIRO Fact Sheet “Piecing together the patches” (AY020605)
- LWA Fact Sheet “Genetic and ecological viability of plant populations in remnant vegetation” (PF020201) Feb 2002

APPENDIX 7.6: Streamline Abstract

Project Title: CPI 10 Genetic and Ecological Viability of Plant Populations in Remnant Vegetation

Objectives

1. Identify and quantify the genetic and demographic factors that affect the viability of plant populations in vegetation remnants. The focus will be on the effects of genetic erosion, inbreeding and pollinator limitation on seed production and seedling fitness. This will involve the integrated use of molecular genetic tools and demographic monitoring to examine four target taxa with varied ecologies.

2. Examine and model the relationships between key genetic and demographic factors affecting viability and remnant vegetation characteristics such as size, disturbance and landscape position.

3. Compare results among four target taxa with varied ecologies to assess how life-history affects the impact of remnant characteristics on population viability.

4. Based on 1-3 generate specific genetic and demographic guidelines for management of remnant populations of the four target taxa and general landscape design principles for major plant life-history types that will maximise the probability of population persistence.

Background

The general aim of this research project was to understand and quantify how genetic and demographic processes interact to influence the viability and long-term conservation value of native plant populations in remnant vegetation and relate this to easily measured landscape and population parameters. This information can then be used to identify and prioritize high viability remnants for in-situ conservation and target them as sources of high quality seed for revegetation and restoration. The project also aimed to test conservation genetics theory regarding the genetic deterioration of small fragmented populations that has been developed based on data from rare plants, and to determine how applicable these paradigms are when dealing with the common species. This is important since it is the more abundant species that are the critical components of landscapes with regard to maintenance of broader ecosystem function such as hydrology and nutrient cycling, as well as provision of habitat for other native organisms.

Methodology

The project combined field-based demographic monitoring, plant growth experiments and the use of molecular genetic markers to determine the effects of three population and landscape parameters: population size, isolation and disturbance on a range of genetic and ecological population response variables that are important for maintenance of population viability: genetic variation, inbreeding, pollination, seed set and seedling fitness. The work was conducted over two years on seven target taxa across two biomes, the fragmented temperate grassland-woodland ecosystems of New South Wales and the variegated kwongan shrublands of Western Australia.

Target taxa were selected to encompass a range of life forms (trees, shrubs and herbs) and a diversity of life-history types including variation in longevity, pollination and dispersal syndrome.

Progress

This project is now complete. Results indicate that common species exhibit a range of genetic and ecological responses to habitat fragmentation that are likely to compromise the long-term viability of remnant vegetation. These include a loss of genetic variation, reduced pollination and seed set and some increase in breeding and reductions in fitness. Key results are that population size is the primary driver of these changes, with isolation affecting some ecological performance criteria such as seed set. The influence of site condition, as measured by disturbance, was not very important. There was significant variation in responses among species and little of this could be predicted based on knowledge of life-history traits. Results of the genetic marker work also point to the importance of interpopulation pollen movement across landscapes in maintaining fecundity in small populations. They also identify increased hybridization as a threatening process in small populations.

Implications

The results of this project indicate that ecosystem fragmentation influences the viability of remnant plant populations through negative effects on both genetic and demographic processes. Based on these results broad guidelines for management of fragmented landscapes to minimize these effects are:

1. Maintain populations bigger than 100-200 reproductive plants when possible. Such populations are critical components of fragmented landscapes because they perform significantly better in terms of reproductive output and have greater genetic diversity and less inbreeding than smaller populations.
2. Minimize isolation distance between populations to maintain biological connectedness through pollen flow and seed dispersal. While population size was consistently the key parameter associated with changes in genetic and demographic processes, isolation also had an influence on pollination and reproductive output.
3. Site condition is not as important as population size or remnant isolation in determining genetic and demographic performance and should be given less consideration in landscape planning.
4. Manage populations within landscapes together over scales of 5-20 km and not as a series of populations independent of other vegetation in the area.

There are a range of critical knowledge gaps in our present understanding of how ecosystem fragmentation influences the viability of remnant plant populations through its effects on ecological and genetic processes. Four key issues stand out as being very important and yet being very poorly understood:

1. The frequency, extent and scale of genetic and demographic connectedness among populations.
2. The influence of life-history traits or ecological “type” on sensitivity to fragmentation.
3. The medium term effects of accumulated inbreeding through biparental mating.
4. The importance of geographical genetic structure within landscapes for adaptive traits and for gross cytogenetic differences.

Keywords

Genetic, demographic, population viability, fragmentation, conservation