ASSESSMENT OF POPULATION GENETIC VARIATION AND STRUCTURE OF *ACACIA WOODMANIORUM*, AND ITS PHYLOGENETIC RELATIONSHIP TO OTHER *ACACIA* SPECIES

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Final report to Karara Mining Ltd by the Department of Environment and Conservation.

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Department of
Environment and Conservation
Our environment, our future

Front cover photographs by Steve Dillon and David Coates.
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EXECUTIVE SUMMARY:
FINAL REPORT

AN ASSESSMENT OF POPULATION GENETIC VARIATION AND STRUCTURE OF *ACACIA WOODMANIORUM*, AND ITS PHYLOGENETICC RELATIONSHIP TO OTHER ACACIA SPECIES.

Phase 1. Assessing levels and partitioning of population genetic variation within *Acacia woodmaniorum* and the evolutionary relationships and distinctiveness of this species to its closest relatives.

1. *The extent and partitioning of population genetic variation within Acacia woodmaniorum*

This section aims to quantify the potential impact of mining scenarios on genetic variation within *Acacia woodmaniorum* and to establish a baseline for future management of genetic variation. Comprehensive germplasm collections were conducted across the range of *A. woodmaniorum*, DNA extraction protocols refined and species specific genetic markers (microsatellites) optimised for use in *A. woodmaniorum*. 573 individuals from 33 populations across the species range were genetically characterised with 15 microsatellite loci and analysis of genetic diversity, population genetic variation, spatial genetic structure and an initial assessment of the potential impact of specific mining scenarios on the species conducted.

A comprehensive report on this component of the project has been provided with the Eighteen Month Report. The main findings reveal,

- Overall genetic diversity within *A. woodmaniorum* is relatively high and comparable to other *Acacia* species (including common species) investigated to date.
- Genetic diversity is concentrated in the main population across Mungada/Windaning ridge. The next greatest level of diversity is found in the Jasper Hills region, followed by the Blue Hills and Terapod populations. Interestingly the Blue Hills population contains a significant number of genetic variants not found anywhere else and more unique variants than do populations in Jasper Hills which is much larger.
- A pattern of increasing genetic difference with increasing geographic separation is found among populations over the species range suggesting that the populations have been stable within this region for a long period of time.
- Isolation by distance is found within the Jasper Hills region, likely due to a restricted level of gene flow among populations associated with the discrete nature of the habitat i.e. the smaller disjunct hematite ranges providing a non continuous habitat.
- There is no isolation by distance among the Mungada/Windaning ridge populations indicating significant pollen movement and gene flow between individuals across this region.
- Genetic differences between populations and regions are low and overall estimates of gene flow via pollen dispersal are relatively high. This result is

unexpected and raises the issue of the pollinators and seed dispersers and their apparent capacity to move over significant distances.

- Mating within populations appears to be random with little evidence of inbreeding.
- An initial assessment of habitat removal on genetic diversity indicates that the removal of plants at Terapod, Blue Hills and the most westerly populations across the main range of Mungada/Windaning ridge would result in:
    1. A relatively small drop (13%) in total species genetic diversity.
    2. A relatively large drop (45%) in overall unique genetic variation that is genetic variation unique to a single location.
- The loss of unique genetic variation is of particular concern for the Blue Hills population.
- Populations that may be impacted upon by mining activities, particularly those on Mungada ridge, may play an important role in the maintenance of gene flow and genetic continuity among populations and regions given their central location within the species range.

Characterisation of microsatellite DNA markers is described in a technical paper published in the journal Conservation Genetics Resources. Results of this component of the project were presented by M. A Millar at the Australian Network for Plant Conservation 8th National Conference (2010) in Perth, WA, at the MEDECOS XII Conference (2011) in Los Angeles, USA, and at the SER2011 World Conference (2011) in Mérida, Mexico. A manuscript that presents a detailed analysis of spatial genetic structure in *A. woodmaniorum* in the context of its disjunct niche habitat has been submitted for publication in the journal Heredity.

2. Evolutionary relationships and distinctiveness of Acacia woodmaniorum to its closest relatives

The *Acacia alata* species complex was originally suggested by taxonomists to be the closest extant relative to *Acacia woodmaniorum*. DNA of each subspecies of the *A. alata* complex and of *A. woodmaniorum* was provided to colleagues at the CSIRO Canberra and the University of Melbourne in order to confirm this. Phylogenetic analysis of these species and others in the Pulchelloidea clade of *Acacia* for a range of sequences including matK, rpl32, ITS and ETS suggested *A. woodmaniorum* was most closely aligned with *A. cerastes* Maslin and *A. restiacea* Benth.

Comprehensive germplasm collections were conducted for *A. alata*, *A. cerastes* and *A. restiacea* with the view of investigating comparative patterns of phylogeography among the closely related species. However, more recently, unpublished results from colleagues suggest that *A. woodmaniorum* is instead most closely related to *A. pterocaulon*. In addition informative variation in chloroplast DNA appears to be limited or not present across the range of *A. woodmaniorum*. The extended part of the project investigating comparative phylogeography requires definitive results from the *Acacia* phylogenetic project conducted at CSIRO and the University of Melbourne. Although we have provided material for this project and have received various progress reports, the findings to date regarding the relatives of *A. woodmaniorum* have been ambiguous and the situation currently remains unresolved. This collaboration will continue and we expect to be able to provide

advice on the evolutionary relationship of *A. woodmaniorum* with its close relatives in the near future.

Phase 2 Determining how key population genetic processes such as mating systems, gene flow and pollination may influence future levels and patterns of genetic variation in *A. woodmaniorum*.

This section aims to determine the level of outcrossing, inbreeding and correlated paternity in *A. woodmaniorum* and the role of the mating system and pollen mediated gene flow in maintaining genetic diversity and genetic connectivity across the species range. It also aimed to determine if mating system parameters and levels of pollen immigration were affected by small population sizes or population isolation. 718 seed pods from across ten populations were assessed for seed production and seed was genetically characterised at nine microsatellite loci for analysis of the mating system and gene flow. The main findings reveal;

- *A. woodmaniorum* is characterised by 12 or more embryos with mature pods containing an average of 6.48 fertilised embryos.
- An average of 68% of initially fertilised embryos develops into viable seed.
- Seed set is low in *A. woodmaniorum* and seed is predated upon by *Coleoptera* sp. and birds or mammals and seed is lost to abortion (24%).
- Correlated paternity within pods suggests that an average of two different fathers sire all seed within a pod.
- The species is predominantly if not obligately outcrossing and appears to have strong selection mechanisms against selfed or inbred seed.
- Small population sizes and degree of population isolation have no impact on mating system parameters.
- Levels of pollen immigration into populations varied from 13% to 61% and averaged 40% for eight small isolated populations of *A. woodmaniorum*.
- Small population sizes and degree of population isolation have no impact on the amount of pollen immigration into populations.
- Results confirm the capacity of the pollen dispersal system to affect a high degree of dispersal into small and isolated populations.
- A highly outcrossed/self incompatible mating system and large effective population sizes are expected to maintain levels of genetic variation in *A. woodmaniorum* into the future.

Results of this component of the project were presented by M. A Millar at the MEDECOS XII Conference (2011) in Los Angeles, USA, and at the SER2011 World Conference (2011) in Mérida, Mexico. A manuscript that presents a detailed analysis of the mating system and pollen mediated gene flow in *A. woodmaniorum* is in preparation for publication.

FINAL REPORT

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1. The extent and partitioning of population genetic variation within *Acacia woodmaniorum*

Introduction

Acacia woodmaniorum is a rare and extremely geographically restricted sprawling, prickly shrub first described in 1999 (Maslin& Buscumb 2007). The species occurs on a series of highly weathered low ranges on the BIFs of the Yilgarn craton. This land system is located on the border of the species-rich Transitional Rainfall Zone and the Arid zone in the Midwest region of Western Australia. Field surveys have shown A. woodmaniorum to be restricted to approximately 29,000 plants with high substrate specificity for the skeletal soils and rock crevices of hematite/magnetite rich outcrops. The geographic distribution of *A. woodmaniorum* covers an area less than 40 km2 and is comprised largely of two discrete banded ironstone ranges, Mungada/Windaning Ridge and Jasper Hills, with two smaller populations at the Blue Hills and Terapod sites (Figure 1). The bulk of plants (less than 25,000) occur across the almost continuous habitat of Mungada/Windaning ridge, with several small (from a single plant to 30 plants) 'outlying' populations occurring on small outcrops of hematite off the main ridge. The habitat of Jasper Hills differs with plants (~3,000 in total) occurring as smaller populations (17 to 1047 plants) on a number of disjunct hematite ranges and smaller outcrops.

The amount of genetic diversity or level of genetic variation within populations of *A. woodmaniorum* and contained within the species overall is a fundamental parameter of the species evolutionary history and conservation biology. High levels of genetic diversity are required in order to maintain the health of individuals and, in the longer term, the ability of populations to respond to forces of selection and a changing environment. In this way, the maintenance of high levels of population genetic diversity acts to maintain the evolutionary potential of species and long term survival.

Loss of population and overall species genetic diversity is predicted with the direct destruction of individuals and of habitat. The degree of loss is related to the severity of habitat destruction and duration of reduced population size. Mining and other activities that include the direct destruction of individuals will result in reduced genetic diversity via the immediate loss of alleles, or allelic richness (the simplest measure of genetic diversity at a locus). The direct destruction of individuals will also result in reduced private allele richness (the number of unique alleles in a

population) which is a measure of genetic distinctiveness of populations. Rare alleles (those that occur at low frequency) will initially be lost from populations, then, more common alleles will be lost.

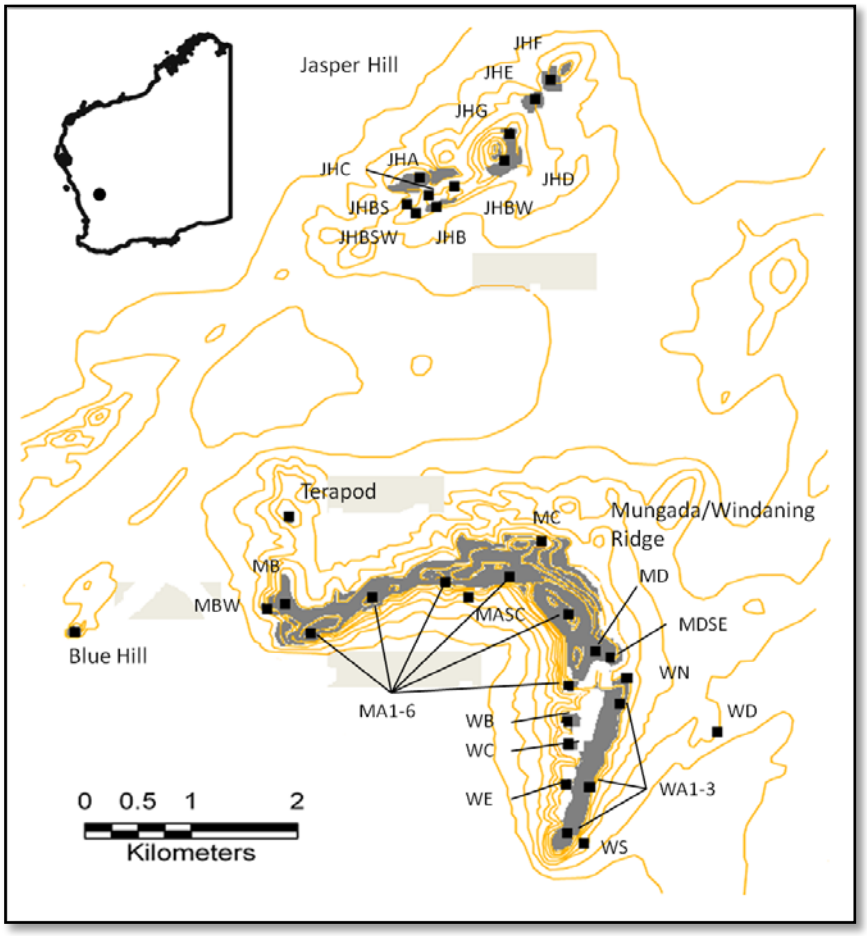


Figure 1 Distribution of *Acacia woodmaniorum* (grey) showing regions and locations of sampled populations (black squares). Note: In earlier reports populations WD and WE were transposed and this error has been corrected here.

Depending on the severity and duration of reduced population size (or genetic bottleneck), genetic diversity may become severely depleted (Ellstrand& Ellam 1993). Decreased population size is likely to correspond to increased geographic distance and isolation between populations and reduced gene flow between populations. Combined with the effects of random genetic drift, allelic loss and reduced levels of gene flow may result in increased levels of genetic differentiation among disturbed populations (Slatkin 1987; Young *et al.* 1996). Small populations may also suffer the negative genetic effects associated with increased levels of inbreeding or mating among related individuals.

Levels of genetic diversity within populations are the result of various processes including forces of selection, genetic mutation, random genetic drift, and the patterns of migration of individuals and of mating. The impacts of various combinations of these forces on *A. woodmaniorum* are unknown and are difficult to determine for a given species. Despite this, broad predictions can be made of the levels and patterns of genetic diversity that may be expected in *A. woodmaniorum*. These

predictions are based largely on the geographical distribution and size of populations and the resulting patterns of migration and mating that may be expected among and within populations. As a rare endemic species with a highly restricted habitat we may expect *Acacia woodmaniorum* to have lower levels of genetic diversity than is found in more widespread and common taxa of the genus. Larger populations are expected to maintain higher levels of genetic diversity and to maintain random patterns of mating. Smaller and more isolated populations are expected to show a degree of genetic impoverishment, lower levels of gene flow or immigration, and a degree of inbreeding due to mating within a restricted number of related individuals
Given the highly specific habitat occupied by *A. woodmaniorum*, and the disjunct nature of the hematite ridges on which it occurs, we expect a degree of genetic differentiation among populations, with increasing levels of genetic differentiation among populations as geographic distance increases.

In this study, we assess the levels of genetic diversity within *Acacia woodmaniorum* and the patterns of genetic differentiation or genetic structure among populations across the species range. We determine a range of parameters describing the patterns of gene flow and genetic connectivity among populations. This information establishes a baseline for future management of *A. woodmaniorum* and gives insight into the processes shaping levels and patterns of genetic diversity in narrow endemic flora of the banded ironstone formations. We also determine the potential impact of several scenarios of population loss on measures of short term diversity loss, including allelic richness and private allele richness, in populations of *A. woodmaniorum*.

Materials and Methods

Sample collection and DNA extraction

Phyllodes were collected from 573 individual plants from across the species range. The sampling of 566 individuals was originally reported but subsequent field trips for seed pod collection revealed 7 additional individuals located in small sites to be used for paternity analysis and gene flow studies. These seven individuals were sampled and the total number of individuals used in the study was 573 (Table 1). Numbers of individuals sampled in each region and population are provided in Table 1. Twenty individuals were sampled at six sites across the large continuous region of Mungada Ridge (MA1-MA6) /Windaning Ridge (WA1-WA3), and from the larger populations on the more disjunct ironstone outcrops of the Jasper Hills region (JHA, JHB, JHD and JHE). Five individuals each were sampled from smaller populations (MB-MD, WC-WD and JHC and JHF-JHG). The smallest 'outlying' populations located off Mungada/Windaning ridge (WD, WE and 'outliers') were exhaustively sampled, as were the Blue Hills and Terapod regions. These sites will be used in future work to delineate patterns of pollen dispersal within the species. The position of all sampled individuals was recorded using a differential Global Positioning Satellite (dGPS) and mapped using Geographical Information Software (GIS) Arcmap™ 9.1 (Figure 1). Fresh phyllode samples were lyophilised and DNA extracted following Millar (2009). All individuals were genotyped using fifteen selected microsatellite primer pairs with PCR conditions previously described for *Acacia woodmaniorum* (Millar 2009). 16 primer pairs were initially selected for genotyping the main germplasm collection however one primer pair (AwC008) failed

to amplify consistently in previously untested populations and its use was discontinued.

Table 1 Region, population and the number of individuals of *Acacia woodmaniorum* sampled.

Region	Population and number of individuals sampled										Total
Mungada Ridge/ Windaning Ridge	MA1-6	MAB	MAC	MAD	WA1-3	WAB	WAC	WAD	WAE	Outliers	
	120	5	5	5	60	5	5	29	16	30	280
Jasper Hills	JHA	JHB	JHC	JHD	JHE	JHF	JHG			Outliers	
	20	20	5	20	20	5	5			43	138
Blue Hills						145 (all)					
Terapod						10 (all)					
Total											573

Statistical analysis

Utility of loci

Microsatellite genotypes were tested for departures from Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) between locus pairs within each region for the 15 loci using exact tests as implemented in the computer program GENEPOP version 4.0 (Raymond& Rousset 1995). Loci are tested for HWE as microsatellite markers are assumed to be neutral genetic markers not affected by the forces of selection. If a locus is not in HWE selection may be acting on allele and genotype frequencies in that population and population genetic structure may be overestimated. It is also important that microsatellite loci not be in LD. LD means two or more loci are located close together on the same chromosome. In this case alleles will not be independent of each other and will be inherited together during meiosis. Thus the information two linked loci provide becomes redundant for one of the loci. Sequential Bonferroni corrections were applied to alpha values in the determination of significance to correct for multiple comparisons of HWE and LD (Rice 1989). Loci were assessed for null alleles, which are undetected alleles that fail to amplify, within populations and for the species overall using GENEPOP (Raymond& Rousset 1995). The presence of null alleles may lead to reduced estimates of allelic richness and overestimation of homozygosity within populations and loci with high frequencies of null alleles are not suitable for population genetic analysis.

Genetic diversity within A. woodmaniorum

Genetic diversity parameters including the total number of alleles, number of private alleles, means of the number of alleles per locus (*A*), the number of effective alleles (*Ne*), the proportion of polymorphic loci (*P*), expected heterozygosity (*He*) and observed heterozygosity (*Ho*), were assessed for all populations within regions, and each of the four regions using the program GenAlEx 6.3 (Peakall& Smouse 2006). To compensate for the bias associated with samples of different size, rarefaction was conducted on measures of allelic richness and number of private alleles (Kalinowski 2004). This statistical procedure removes bias by standardising for

sample size and was implemented using the algorithms in the computer program HP-RARE (Kalinowski 2005). Sampling was standardised to 20 genes per population with one population per region. Levels of genetic diversity were hierarchically partitioned using the Analysis of Molecular Variance (AMOVA) method implemented in GenAlEx. Analysis was conducted on F_{ST} values at the individual, population and regional level. Statistical testing was conducted using 999 random permutations.

Genetic differentiation among populations

Genetic differentiation among regions of *A. woodmaniorum* was investigated on the basis of allele identity using pairwise estimates of the intra-class kinship coefficient of Weir and Cockerham (F_{ST}, 1984), and on the basis of differences in allele size assuming stepwise mutation using pairwise R_{ST} values (Slatkin 1995). Both F_{ST} and R_{ST} are measures of the proportion of total genetic diversity that separates populations. Low values of F_{ST} suggest little genetic differentiation between populations and, if all populations were in HWE with the same allele frequencies, values equal zero. Pairwise values of F_{ST} and R_{ST} were estimated among regions using GENEPOP. The overall F_{ST} among regions, among all populations, and among populations within each multi-population region (Jasper Hills and Mungada/Windaning ridge) was also estimated using GDA (Lewis& Zaykin 2001) and 95% confidence intervals obtained with 1000 bootstraps.

In order to more clearly visualise the levels of genetic differentiation among populations, the ordination technique of multivariate principal component analysis was also conducted. Covariance genetic distance matrices were constructed by AMOVA on allele frequencies (Excoffier *et al.* 1992), with 99 randomization steps, using GenAlEx. The matrices were then ordinated in a three dimensional space by principal component analysis (PCA) using a standardised data set and plotted using Statistica software (StatSoft 2001). Analysis was conducted on both regional and population scales.

Genetic differentiation among populations across the species range was also investigated using the more complex Bayesian method of Pritchard et al. (2000) and Falush et al. (2003), as implemented in the program STRUCTURE version 2.3. This method identifies genetically distinct clusters (*K*) based on allele frequencies. Runs to determine K were conducted with a burn-in period of 10 000, followed by 100 000 iterations. An ancestory model incorporating admixture and correlated allele frequencies was used. This method of analysis allows for the provision of spatial information on populations and we ran the model using prior information on population origin. Ten runs were conducted for each K from K = 1 to K = 6 and the second order rate of change in the estimated Ln probability of the data (ΔK), determined following Evanno et al. (2005), and using the program Structure Harvester v0.56.3 (Earl 2009). ΔK was then plotted against K to determine the optimal number of clusters. The mean value (Q) of the proportion of membership of individuals (q_i) in each cluster was obtained.

Spatial genetic structure

In order to investigate isolation by distance, Mantel tests were conducted for multi-population regions as well as for all populations across the species range, using GenAlEx 6.3. Linear regression was conducted on matrices of pairwise values of linearised F_{ST} ( ) and of the log geographic distance (Rousset 1997; Slatkin 1993). Significance tests for correlation were conducted using 999 random

permutations (p(r_{xy}-random ≥ r_{xy}-data)(Smouse& Long 1992; Smouse *et al.* 1986). In order to investigate the distances over which spatial genetic structure occurs, we conducted linear regression of pairwise population F_{ST}, on geographic distance classes using SPAGeDI 1.3. We conducted significance tests using 10 000 permutations for F_{ST} matrices on 8, 10 or 12 Euclidian distance classes calculated from spatial coordinates. The most appropriate number of distance classes was selected by comparing the proportion of all populations represented in each class and the coefficient of variation of the number of times each population was represented in that distance class. Analysis was conducted for all populations across the species range and for populations within Jasper Hills.

Mating patterns and population sizes

The fixation index (F), a measure of departure from random mating due to inbreeding, was assessed for all populations within regions, and for each of the four regions using GenAlEx. Values of population genetic differentiation (F_{ST}) were converted to the number of migrants following Slatkins island model.

In populations with recently reduced numbers, the level of allelic diversity is reduced faster than the level of expected heterozygosity, and expected levels of heterozygosity will be greater than levels of heterozygosity that may be expected under drift/gene flow equilibrium (Cornuet& Luikart 1996). To determine whether populations have experienced recent reductions in size, we tested for expected heterozygotic excess at a regional scale as implemented in Bottleneck 1.2.0.2 (Piry *et al.* 1999). Equilibrium conditions were simulated using 1000 replications assuming a Two-phased model of mutation (TPM) and significance tested using Wilcoxon signed-rank tests.

Impacts of various scenarios

In order to assess the impact of direct destruction of plants of *A. woodmaniorum* on short term levels of genetic diversity, we re-determined measures of allelic richness, private allele richness and heterozygosity for various scenarios of population destruction and compared the results to the baseline data. We assessed five scenarios a) the loss of ten plants at Terapod, b) the loss of the Blue Hills population and c) the loss of the three most westerly populations of the main range (MA1-3) d) the loss of the three most westerly populations of the main range and all plants at MB and MBSW e) the loss of all above mentioned populations. These scenarios were chosen as it seems likely these populations may be at greatest risk of removal by mining activities.

Results

Utility of loci

Fourteen of the fifteen microsatellite loci were polymorphic in all populations of *A. woodmaniourm*. Locus AwD012 was monomorphic in Terapod but polymorphic in other populations. A total of 199 alleles were detected across 15 loci, ranging from a maximum of 23 alleles in AwB109 to a minimum of 5 alleles in AwD010. After adjusting for multiple comparisons (n = 495) significant departures from HWE, in the form of heterozygote excess, were observed for locus AwC001 in most sampling

locations across the main range (MA1, 3, 4, 5, 6 and WA1, 2), some larger populations (JHA, JHB, JHD, JHE and Blue Hills), and in two smaller populations (WD and JHBS). Significant departures from HWE in the form of heterozygote deficiency were observed for one or two loci in populations JHB (AwB003), MA1, MA2, MA4 and WA2 (AwB109), WA3 (AwB107), WD (AwA129 and AwB109), and JHBS (AwB003 and AwB009) and for nine loci in the Blue Hills population (AwA124, AwA129, AwD008, AwB109, AwD010, AwB001, AwB003, AwB107 and AwD012). After correcting for multiple comparisons (n = 3465) significant LD was detected for one locus pair combination (Aw124 and AwD010) in the Blue Hills population. Estimates of Null allele frequencies were low (<0.010) for most loci, reaching a maximum of 0.2441 for D010 in Blue Hills. Given the lack of significant LD and low estimates of the frequency of null alleles we conclude that the loci are suitable for the analysis of population genetic diversity and structure.

Genetic diversity within *A. woodmaniorum*

The total number of alleles observed in regions for *A. woodmaniorum* ranged from a maximum of 171 alleles across Mungada/Windaning ridge to a minimum of 62 alleles for the ten plants at Terapod. The mean number of alleles per locus for populations within regions ranged from a maximum in Blue Hills (8.53) to a minimum in Jasper Hills (3.96, Table 2). At a regional level the mean number of alleles per locus was greatest across Mungada/Windaning ridge (11.40) and lowest for Terapod (4.133). The mean number of effective alleles per locus showed a similar pattern, being highest for Blue Hills (3.102) and lowest for populations in Jasper Hills (2.451), and highest for Mungada/Windaning ridge (3.784) and lowest for Terapod (2.806) at a regional level. The proportion of polymorphic loci was greatest for Blue Hills and lowest for Terapod.

Measures of allelic richness within populations are biased by the number of individuals sampled from each population, as well as the number of populations sampled from each region. Larger populations or populations in which more individuals are sampled are expected to contain more alleles than smaller populations or single individuals. Regions with more populations or sampling locations are also expected to hold more allelic variation than regions with fewer populations. In order to remove this bias rarefaction was conducted on measures of allelic diversity per locus. When the number of alleles per locus within regions were standardised to one population with 20 genes, the number of alleles observed remained greatest for the main population across Mungada/Windaning ridge (5.13) and lowest for Terapod (4.13).

Another measure of genetic diversity at microsatellite loci is the expected heterozygosity (H_e) or overall gene diversity. Values of expected heterozygosity were similar for all regions, but again, greatest for the main range across Mungada/Windaning ridge (0.627, Table 2) and lowest at Terapod (0.564).

Comprehensive studies of genetic diversity using microsatellite markers have been conducted for few Acacia taxa, despite their ecological, cultural and economic importance. Levels of microsatellite genetic diversity within *A. woodmaniorum*, including the number of alleles per locus, effective number of alleles per locus, percentage of polymorphic loci, and observed and expected heterozygosities, are

however all greater than those found in a comprehensive study of *A. saligna*, a common species with a widespread distribution across south western Australia (Millar *et al.* 2011).

Table 2 Microsatellite diversity in *Acacia woodmaniorum*. Means are shown for the number of individuals sampled (*N*) and genetic diversity parameters including the number of alleles per locus (*A*), the number of effective alleles (*Ne*) the proportion of polymorphic loci (*P*), expected heterozygosity (*He*), observed heterozygosity (*Ho*) and the fixation index (F) for populations within regions and for regions. Values in parenthesis are standard errors.

Region		*N*	*A*	*Ne*	*P*	*He*	*Ho*	F
Jasper Hills	Populations	13.533 (0.599)	3.960 (0.176)	2.451 (0.100)	96.00 (2.67)	0.510 (0.017)	0.536 (0.023)	-0.050 (0.030)
	Region	135.33 (0.637)	7.933 (1.071)	2.984 (0.414)	100.00 (0.00)	0.588 (0.044)	0.522 (0.054)	-
Mungada/ Windaning Ridge	Populations	11.575 (0.401)	4.317 (0.130)	2.759 (0.084)	94.60 (1.99)	0.537 (0.012)	0.527 (0.016)	0.004 (0.024)
	Region	243.07 (8.759)	11.40 (1.264)	3.784 (0.660)	100.00 (0.00)	0.627 (0.051)	0.521 (0.049)	-
Blue Hills	Populations	145 (0.00)	8.533 (1.162)	3.102 (0.574)	100.00 (0.00)	0.573 (0.051)	0.509 (0.061)	0.106 (0.080)
	Region	134.13 (3.070)	8.533 (1.162)	3.102 (0.574)	100.00 (0.00)	0.573 (0.051)	0.509 (0.061)	-
Terapod	Populations	10 (0.00)	4.133 (0.496)	2.806 (0.303)	93.33 (0.00)	0.564 (0.057)	0.533 (0.067)	0.043 (0.076)
	Region	9.6 (0.273)	4.133 (0.496)	2.806 (0.303)	93.33 (0.00)	0.564 (0.057)	0.533 (0.067)	-
All populations		15.822 (0.997)	4.331 (0.111)	2.677 (0.064)	95.15 (1.49)	0.531 (0.010)	0.529 (0.013)	-
Species overall		522.133 (11.790)	13.267 (1.631)	3.781 (0.709)	100.00 (0.00)	0.6431 (0.049)	0.516 (0.049)	0.159 (0.071)

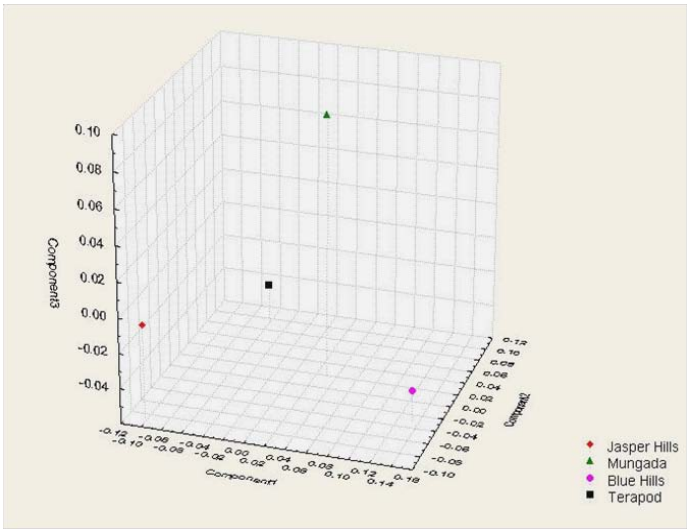
Genetic differentiation among populations

A measure of the distinctiveness of populations is the number of private alleles they contain. Private alleles are alleles present in a given population and not found in any other population. Private alleles were abundant in populations of *A. woodmaniorum*, being detected in 24 of the 33 sampling locations, with the exceptions being JHBW, JHC, JHD, JHF, MA4, MASC, MDSE, WN and WS. All regions contained private alleles; 28 were detected across the main Mungada/Windaning ridge, 12 alleles were unique to Blue Hills, 8 alleles were unique to the Jasper Hills region, and one unique allele was observed at Terapod. The number of private alleles within populations can also be expected to be biased by the number of individuals sampled from each population and the number of populations sampled from each region. However, when rarefaction was conducted on the number of private alleles per region for *A. woodmaniorum*, the number of private alleles remained greatest for Mungada/Windaning ridge (17.4), decreasing through Blue Hills (13.4) and Jasper Hills (11.8) to Terapod (6.4). Values for sampled populations within each region did change (data not presented).

F_{ST} is another measure of the genetic distinctiveness of populations, based on allele identity or allele frequency. A low level of genetic differentiation is evident among

populations of *A. woodmaniorum* across the species range (F_{ST} = 0.0977) and genetic differentiation at a regional scale was lower (F_{ST} = 0.0677). The Blue Hills population shows the greatest degree of genetic differentiation from the other regions (Table 3). On a regional scale, genetic differentiation is greatest between Jasper Hills and Blue Hills (F_{ST} = 0.109), low between Jasper Hills and Terapod (F_{ST} = 0.056) and lowest between Jasper Hills and the main range on Mungada/Windaning ridge (F_{ST} = 0.048). This pattern of genetic differentiation is not surprising, as divergence in allele identity or frequency is a measure of genetic drift among populations and greater levels of genetic drift and genetic differentiation are expected between populations that are more geographically isolated from each other. Populations in the Jasper Hills region are located geographically furtherest from Blue Hills (approximately 5.75km, see Table 4), closer to the main range (4.12 km), and closest to those at Terapod (3.64 km). The population at Blue Hills is most genetically similar to its geographically closest population - the main range of Mungada/Windaning ridge (F_{ST} = 0.061, 1.87 km), and plants at Terapod are most genetically similar to their closest population which is the main range on Mungada/Windaning ridge (F_{ST} = 0.050, 0.66 km).

a



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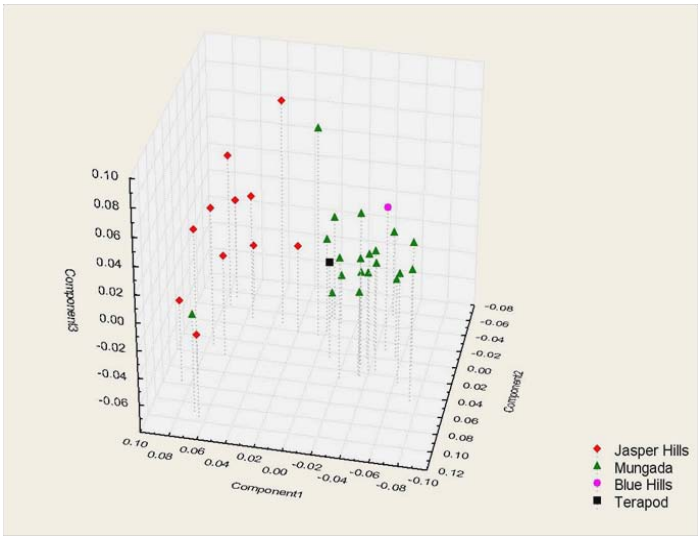


Figure 2. Principle component analysis showing a) the genetic relationships among *Acacia woodmaniorum* a) regions and b) populations.

Table 3. Pairwise F_{ST} values below the diagonal and R_{ST} values above the diagonal for regions of *Acacia woodmaniorum*.

	Jasper Hills	Mungada/Windaning	Blue Hills	Terapod
Jasper Hills	-	0.132	0.186	0.106
Mungada/Windaning	0.048	-	0.046	0.092
Blue Hills	0.109	0.061	-	0.121
Terapod	0.056	0.050	0.097	-

Genetic differentiation among populations is further illustrated in the PCA diagrams (Figure 2). At a regional scale, the first three principal components explained 100% of the variation seen; component 1 53.34%, component 2 23.38% and component 3 18.32% (Figure 2a). By populations the first three principal components explained 71.86% of the variation seen; component 1 37.69%, component 2 21.56% and component 3 12.62% (Figure 2b).

Genetic differentiation among regions was also calculated based on allele size (R_{ST}, Table 3). This measure indicates the relative contribution of stepwise mutation to genetic divergence among populations. Most pairwise population estimates of R_{ST} were greater than estimates of divergence based on allele identity, indicating that stepwise mutation in microsatellite repeats may contribute more to genetic differentiation among regions of *A. woodmaniorum* than random genetic drift does. On the basis of allele size, genetic differentiation is again greatest between Blue Hills and Jasper Hills (R_{ST} = 0.186). Jasper Hills and Mungada/Windaning ridge show greater levels of genetic differentiation due to mutation than due to drift however, and Blue Hills and Mungada/Windaning ridge, as well as Blue Hills and Terapod show lower levels of differentiation due to mutation than due to drift.

Hierarchically, the majority of genetic diversity in *A. woodmaniourm* occurs within individuals. Sixty nine percent of all genetic variation is contained within populations, 24% is contained among populations and 7% is contained among regions. Lower genetic diversity among populations and regions is reflected in the relatively low values of pairwise estimates of differentiation among populations and regions. These results suggest that significant levels of gene flow via pollen dispersal are maintained, and indeed estimates of the number of migrants among populations and regions are high.

Genetic differentiation among populations across the species range was also investigated using the complex Bayesian clustering method. This technique clusters populations on the assumptions that genotypes are at HWE, loci are not in LD and a minimum of between cluster genetic differentiation is present, that is, allele frequencies are cluster specific. Bayesian analysis optimally placed populations into one of two clusters representing Jasper Hills, Mungada/Windaning ridge and Terapod combined (cluster 1), or Blue Hills (cluster 2). Values of mean estimated Ln probability of the data (LnP(D)) rose steadily with K until K = 3, after which standard errors about the mean increased (Figure 3a). A plot of ΔK against K revealed a peak at K = 2 and this was taken as the optimal number of clusters (Figure 3b). Figure 3c shows a cluster plot of individuals grouped into one of the two clusters with average

proportion of membership values (Q) of 0.900 for Jasper Hills, Mungada/Windaning ridge and Terapod, and of 0.874 for Blue Hills. A number of populations across Mungada/Windaning ridge (MA1-4, MB, MBW, MC, MA6, WA2, WC and WE) showed some genetic affiliation to Blue Hills (maximum Q for Blue Hills was for MABW, Q = 0.329 and the maximum q_i was for an individual from MA2, q_i = 0.625).

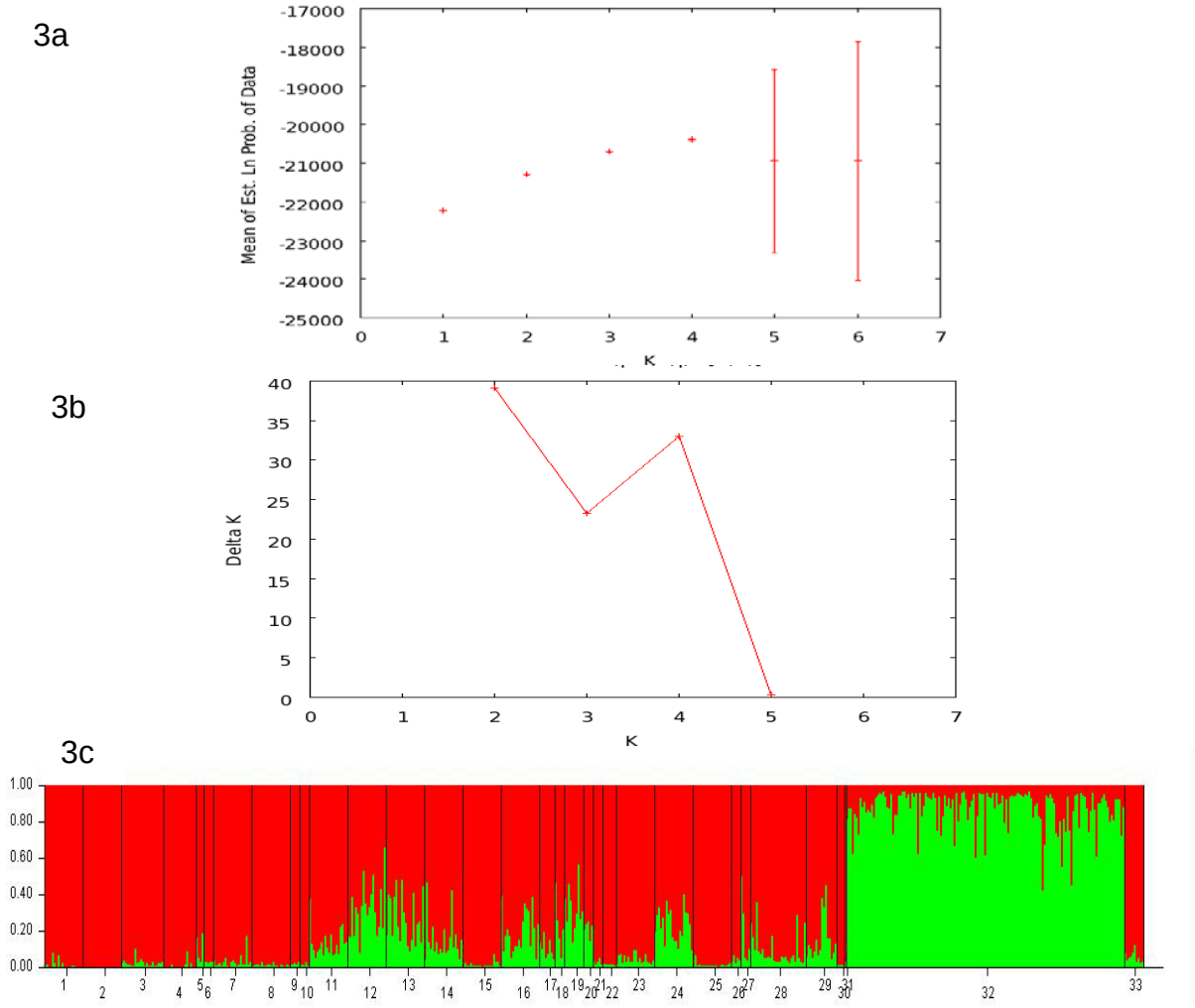


Figure 3 STRUCTURE analyses of sampled individuals of *Acacia woodmaniorum*. a) plot of Ln P(D) against increasing K b) plot of Evannos delta K with increasing K showing a maximum at K = 2 and c) a cluster plot showing the proportion of all sampled individuals in each of two clusters. Populations 1 to 10 are from Jasper Hills, populations 11 to 31 are from Mungada/Windaning ridge, population 32 is Blue Hills and population 33 is Terapod.

Spatial genetic structure

Correlation between genetic and geographic distance was evident for populations across the entire species range ($p = 0.001$, $R^2 = 0.0688$, Figure 4a), suggesting extant populations have persisted long enough to achieve equilibrium between drift and gene flow across the species distribution. Isolation by distance was revealed within the Jasper Hills region ($p = 0.003$, $R^2 = 0.222$, Figure 4b) suggesting that in this region the species has had sufficient time to achieve an equilibrium between the effects of genetic drift and gene flow. In Jasper Hills the influence of limited gene flow spreads to all degrees of geographical separation. This result may be attributed to landscape features, namely the disjunct nature of the ironstone ridges in this area that may act to inhibit gene flow via pollen or seed dispersal among populations. At

a regional scale, a lack of correlation between genetic and geographic distance was detected across the species main distribution on Mungada/Windaning ridge ($p = 0.075$, $R^2 = 0.0233$, Figure 4c), suggesting this large, relatively continuous population is not in drift/gene flow equilibrium. The lack of isolation by distance across the main ridge suggests that that gene flow plays a greater role in shaping genetic structure on the ridge than random genetic drift does and that dispersal via gene flow is not limited in this relatively homogeneous population.

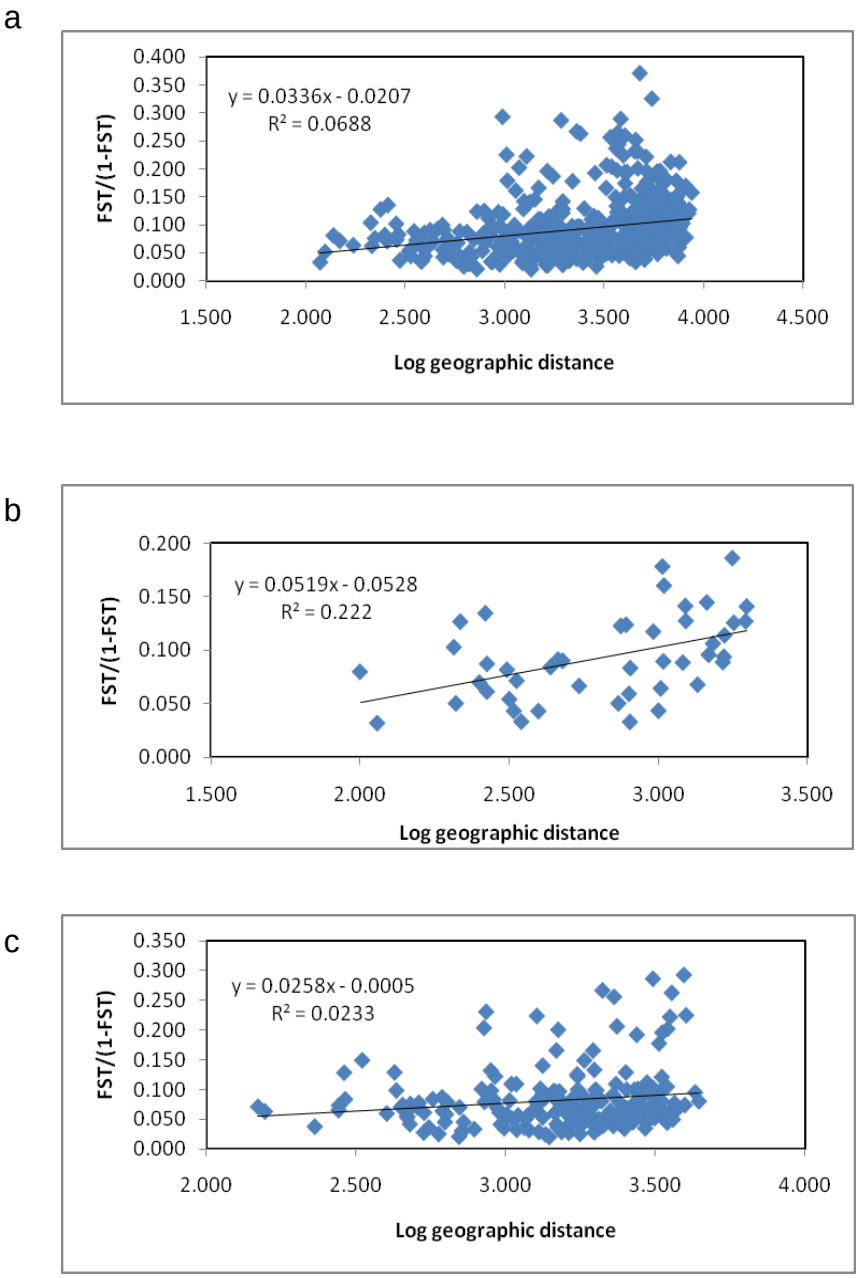
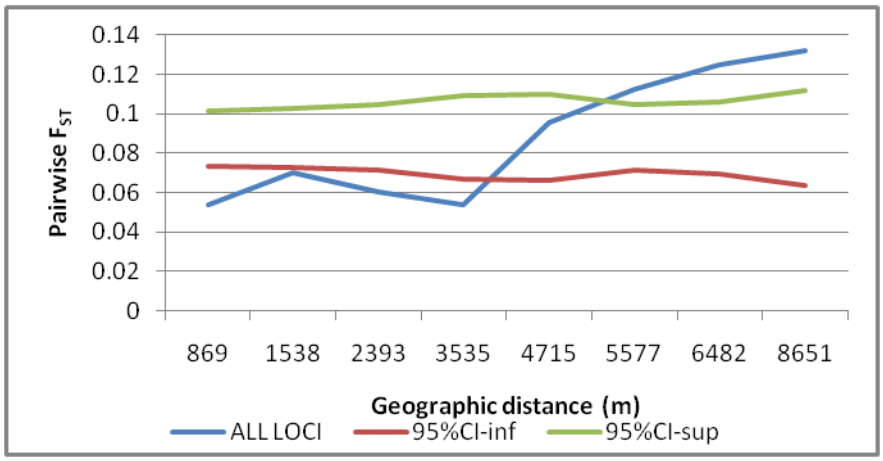


Figure 4. Isolation by distance among (a) all populations of *Acacia woodmaniorum* and (b) among populations within the Jasper Hill and (c) Mungada/Windaning ridge regions. Multilocus estimates of pairwise differentiation are plotted against logarithm of geographic distances.

Spatial autocorrelation analysis of populations across the species range revealed significant correlation of genetic distance and geographic distance at seven of eight optimal geographic distance classes (Figure 5a). Correlation was not significant at

distances of 3535 m to 4715 m. Within Jasper Hills, autocorrelation over eight optimal distance classes revealed significant correlation at distances of 307 m to 398 m and at distances of 1540 m or greater ($p < 0.05$).

a



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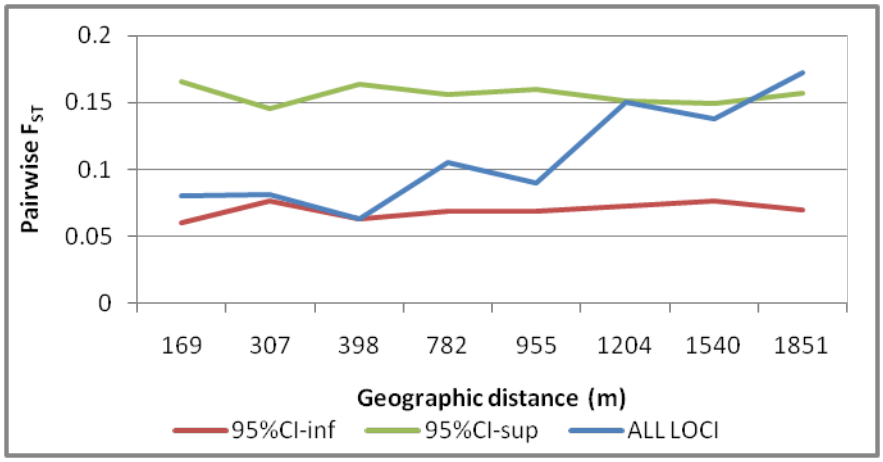

Figure 5. Spatial autocorrelation of a) all sampled populations and b) populations within the Jasper Hills region of *Acacia woodmaniorum*.

Estimates of the fixation index indicate random mating with little inbreeding within populations and regions and this result also suggests that pollen dispersal is not limited in *A. woodmaniorum* (Table 2). Values of the fixation index close to zero indicate random mating with little inbreeding or negative assortative mating within *A. woodmaniourm.* Values of the fixation index close to zero are expected under random mating, with substantial positive values indicating a degree of inbreeding, or breeding among related individuals and negative values indicating a degree of negative assortative mating or heterotic selection.

Random mating with a degree of pollen flow acting to maintain homogeneity among populations is also evident by the high estimates of the number of migrants per generation (N_m). These values are directly related to pairwise estimates of F_{ST} and so to the geographic distance between populations. Regional pairwise values of Nm and of geographic distance between populations are provided in Table 4. Overall 2.31 individuals per generation are estimated to result from migration of either pollen

or seed among populations. On a regional scale 3.44 migrants are estimated per generation.

Table 4. Pairwise N_m values below the diagonal for regions of *Acacia woodmaniorum*. Pairwise geographic distances in kilometers above the diagonal.

	Jasper Hills	Mungada/Windaning	Blue Hills	Terapod
Jasper Hills	-	4.12	5.75	3.64
Mungada/Windaning	4.96	-	1.87	0.660
Blue Hills	2.04	3.85	-	2.42
Terapod	4.21	4.75	2.33	-

No evidence of heterozygotic excess was found within regions of *A. woodmaniorum* indicating that population sizes have not been recently reduced and that populations are at mutation/drift equilibrium. A mode shift in allele sizes was detected for plants at Terapod but this is likely to be a result of small population size for which the qualitative method of analysis is unreliable.

Impacts of various scenarios

The distribution of genetic variation measured as allelic richness (number of alleles) and private allele richness (alleles unique to a region) is shown in Fig. 6. The removal of habitat and therefore plants will negatively impact levels of genetic diversity, including levels of allelic richness, private allele richness and heterozygosity within *A. woodmaniorum* (Table 5). Analysis of baseline data after the removal of certain populations reveals that the loss of the ten plants at Terapod will result in a reduction of 0.05% of the species total allelic diversity and one allele private to that population or 2.04% of private alleles. Loss of the Blue Hills population will reduce total allelic diversity by 6.03% and result in the loss of 24.49% of private alleles; the loss of the three most westerly populations of the main range (Mungada – Windaning Ridge, MA1-3) will reduce total allelic diversity by 4.02% and result in the loss of 14.29% of private alleles. If all of the listed populations (Table 5) are removed, 12.56% of the species total allelic diversity will be lost as will 44.90% of private alleles. This represents a significant amount of the species diversity and may impact on its ability for future adaptation and persistence although this is difficult to quantify. Levels of heterozygosity will be reduced slowly with the loss of increasing numbers of individuals due to the high level of polymorphism in the markers. The populations targeted in the above scenarios are located from the western margin to the center of the species distribution. If large numbers of plants are removed from these central locations this may impact not only levels of genetic diversity but also the maintenance by pollen flow among populations as populations become increasingly isolated.

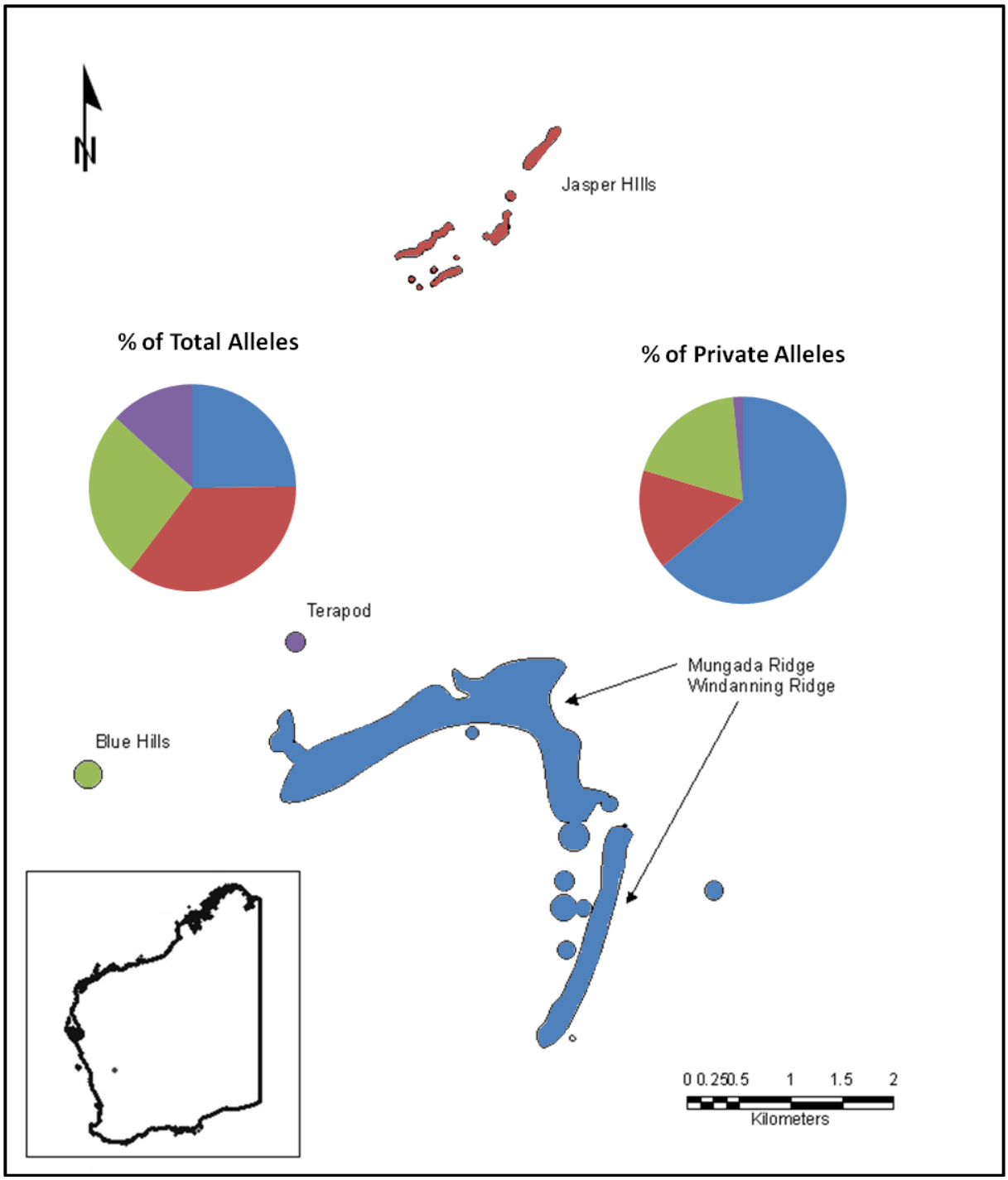

Figure 6. Allelic diversity (number of alleles) and number of private alleles (alleles only found in that region) for the four regions Mungada- Windanning Ridge, Blue Hills, Terapod, and Jasper hills

Table 5. Predicted microsatellite diversity in *Acacia woodmaniorum* under different scenarios of population loss. Genetic diversity parameters are provided for the species after removal of the given population. Parameters include the total number of alleles remaining (At) and the percentage of species allelic diversity lost (At%), the number of private alleles remaining (Ap) and the percentage of private alleles lost (Ap%), and the new values of expected heterozygosity (*He*), and observed heterozygosity (*Ho*). Values in parenthesis are standard errors.

	At	*At% lost*	*Ap*	*Ap% lost*	*He*	*Ho*
Extant Species	199		49		0.631 (0.049)	0.516 (0.049)
Population removed						
Terapod	198	0.05	48	2.04	0.630 (0.049)	0.515 (0.049)
Blue Hills	187	6.03	37	24.49	0.627 (0.049)	0.519 (0.048)
MA1-3	191	4.02	42	14.29	0.629 (0.049)	0.514 (0.049)
MA1-3, MB, MBW	189	5.025	40	18.37	0.631 (0.31)	0.516 (0.49)
Terapod, Blue Hills and MA1-3, MB, MBW	174	12.56	27	44.90	0.625 (0.049)	0.519 (0.048)

Conclusion

Despite its restricted distribution and extreme habitat specificity, *A. woodmaniorum* is not genetically depauperate, as may have been expected for a narrow range endemic, and the species shows high levels of polymorphism at microsatellite loci. The majority of genetic variation is contained within populations and is concentrated in the main populations across Mungada/Windaning ridge, followed by the Jasper Hills region, the Blue Hills and the Terapod populations. This follows expectations of larger populations maintaining greater levels of genetic diversity. A pattern of increasing genetic differentiation with increasing geographic separation is found over the species range and among populations within the Jasper Hills region. Despite this, genetic differentiation among populations and regions is generally low and gene flow via pollen dispersal among populations appears to be high. Populations show little evidence of inbreeding or negative assortative mating and there is no genetic evidence of recent population bottlenecks suggesting habitat destruction, disturbance or anthropogenic fragmentation has not occurred. Populations likely to be impacted by mining operations contain a high degree of genetic diversity and private allele richness and may also be important for the maintenance of gene flow and genetic continuity among populations and regions given their geographic location. This should also be considered in assessing the impact of likely mining scenarios and shall be investigated further in specific studies of pollen flow.

2. Evolutionary relationships and distinctiveness of *Acacia woodmaniorum* to its closest relatives

Introduction

The *Acacia alata* species complex was originally suggested by taxonomists to be the closest extant relative to *Acacia woodmaniorum*. We conducted extensive collections of all four variants of the *A. alata* species complex and provided DNA of each variant of *A. alata* and of *A. woodmaniorum* to colleagues at the CSIRO Canberra and the University of Melbourne to confirm this. Phylogenetic analysis of these species and others in the Pulchelloidea clade of *Acacia* for a range of sequences including matK, rpl32, ITS and ETS suggested *A. woodmaniorum* was most closely aligned not with *A. alata* but with *A. cerastes* Maslin and *A. restiacea* Benth (Gillian Brown, University of Melbourne, personal communication).

In order to extend this part of the project we planned to conduct a comparative phylogeographic study for *A. woodmaniorum*, *A. cerastes* and *A. restiacea*. We subsequently conducted germplasm collections and DNA extractions for *A. cerastes* and *A. restiacea*. *A. cerastes* is a priority one species with a restricted distribution occurring only on White Wells, Ninghan and Mount Gibson stations in the Midwest (Figure 6a). *A. restiacea* is a widespread common species with a distribution from Kalbarri south to Broome Hill and east to Hospital Rocks (Figure 6b). All populations of *A. cerastes* and seven populations of *A. restiacea*, covering the species' range, were sampled. Testing for among population and among species variability for various chloroplast DNA sequences (Shaw *et al.* 2005; Shaw *et al.* 2007) was conducted in these species in order to assess comparative phylogeographic patterns among the species.

More recent (unpublished) results from colleagues at the CSIRO Canberra and the University of Melbourne now suggest that the closest relative to *A. woodmaniorum* is in fact *A. pterocaluon*. Further work in the area of comparative phylogeography requires the accurate determination of the closest relative to *A. woodmaniorum*.

Materials and Methods

Sample collection and DNA extraction

Germplasm was collected from five individuals from 21 populations of *A. alata* (112 individuals) across the species range, from Geraldton south to Albany. Germplasm was collected from two to five individuals from 12 populations of *A. cerastes* (54 individuals) across the species range, and from seven populations of *A. restiacea* (34 individuals) from Kalbarri south to Gidgegannup. DNA has been extracted from all individuals following Millar (2009). Initial testing of DNA sequences for variability among populations and among species was undertaken for *A. woodmaniorum*, *A cerastes* and *A. restiacea*. Chloroplast sequences trnV-ndhC, ndhF-trnL, trnS-trnG, psbA-trnH, psbD-trnT were selected for initial testing based on preliminary tests for variability in *Acacia acuminata*. Methods follow Shaw et al (2005). Testing for sequence variation was initially conducted using two individuals from two populations

of *A. acuminata* as positive controls, two individuals from two populations of *A. woodmaniorum*, two individuals from two populations of *A. cerastes*, two individuals from two populations of *A. restiacea* and two individuals from one population of *A. alata* as an outgroup. Raw sequencing files were manually edited using the BioEdit software version 7.0.5.3 (Hall 1999) and aligned using ClustalW software version 2.0 (Larkin *et al.* 2007).

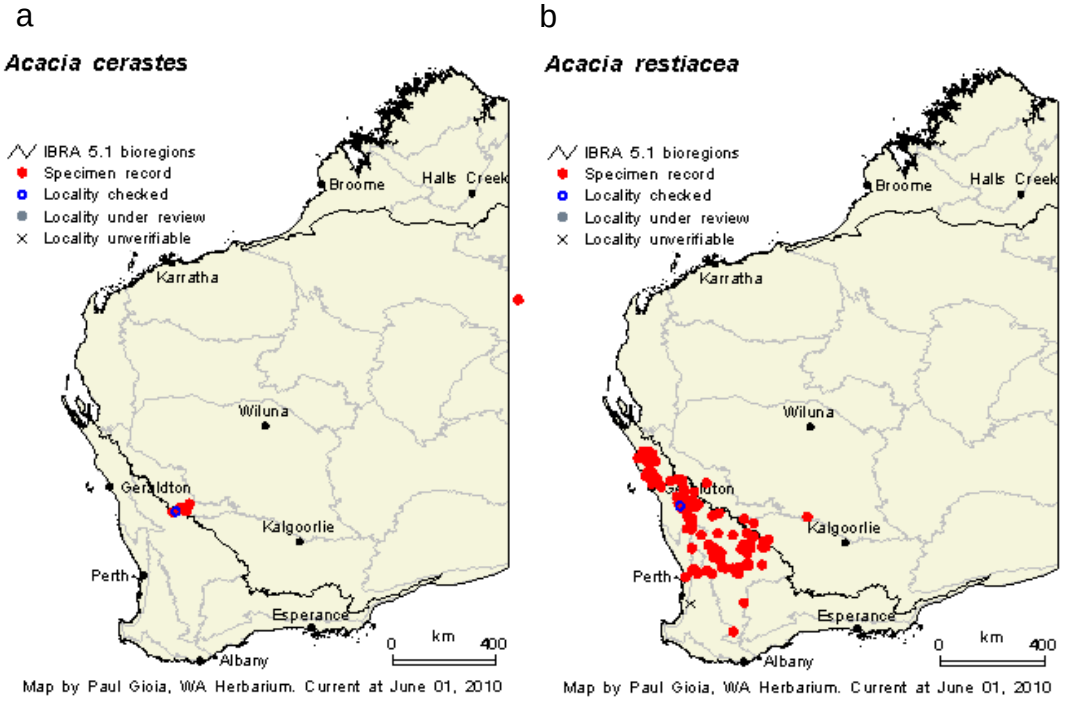

Figure 6 Populations of a) *Acacia cerastes and a) Acacia restiacea*.

Figure 7. *A. cerastes* growing in typical habitat on granite rocks, Beanthiny Hill, Mount Gibson Station. Photograph by M.A. Millar.

Results

Sequencing of chloroplast regions

The following chloroplast regions were tested for amplification and success of sequencing; ndhF-trnL, psbA-trnH, trnS-trnG, trnV-ndhC, psbD-trnT. Preliminary sequencing results are reported in Table 6.

96 samples consisting of 36 *A. woodmaniorum*, 26 *A. restiacea*, 29 *A. cerastes* and 4 *A. alata* samples have been sequenced for trnV-ndhC (both directions), atp-ndhA (atpF) and psbD-trnT (psbD). These sequences have been fully edited and aligned. While variation is present among species, no informative variation has been detected within *A. woodmaniorum*.

Conclusion

Preliminary analysis of three chloroplast regions suggests that informative intraspecific variation is limited or not present within *A. woodmaniorum*. This extended comparative phylogeography component of Phase 1 of the project was resource dependent and continued as far as possible. More recent (unpublished) results from colleagues at the CSIRO Canberra and the University of Melbourne suggest that the closest relative to *A. woodmaniorum* is in fact *A. pterocaluon*. This collaboration will continue and we expect to be able to provide advice on the evolutionary relationship of *A. woodmaniorum* with its close relatives in the near future. However, further planned work in the area of comparative phylogeography will not continue given the expiration of time for this project.

Table 6. Result of sequencing trials for *Acacia* species.

Region	Primer	Length (bp)	Intraspecific Variation in *A. woodmaniorum*	Other comments
ndhF-trnL	ndhF			Primer/ template ratio needs optimising
ndhF-trnL	trnL	<600	None	Microsatellite @ 400bp in *A. alata*
psbA-trnH	psbA	400	None	polyA @ 400bp for all species
psbA-trnH	trnH			Primer/ template ratio needs optimising
trnS-trnG	trnS	200	None	polyA @ 200bp in all species
trnS-trnG	trnG		None	PolyT @ 250bp in all species
trnV-ndhC	trnV	750	Possibly 2 snps	works well
trnV-ndhC	ndhC	200-370	None	polyA @ 210bp in *A. woodmaniorum* polyA @ 370bp in *A. restiacea*
			None	polyA @ 350bp in *A. cerastes*
psbD-trnT	psbD	750	None	works well
psbD-trnT	trnT	1000 for *A. alata*	None	polyT at the start in *A. alata* Fails for *A. restiacea*
trnQ-rps16	trnQ			PCR fails for alata and restiacea
trnQ-rps16	rps16			PCR fails for alata and restiacea
petB	sak25F	386		woodmaniorum and acuminata failed, alata, restiaceae and cerastes give low mixed signals, secondary structure or needs more template
rpl16	sak16F	496		works for acuminata only, low mixed signals for others, more than one binding site
petD	sak19F	298		mixed signals for alata, woodmaniorum and acuminate, secondary structure or needs more template
atpF	sak22R	400-660	None	works well
ndhA	sak28R	~600	None	poly T @ 500 in cerastes

Phase 2 Determining how key population genetic processes such as mating systems, gene flow and pollination may influence future levels and patterns of genetic variation in *A. woodmaniorum*.

Introduction

Results from Phase 1 of the project suggest that high levels of gene flow are maintained among populations of *A. woodmaniourm*. Levels and patterns of gene flow via pollen are linked to the mating system where they influence levels of mating among individuals and levels of selfing and outcrossing within populations. Gene flow and mating systems in turn are affected by pollinators and seed dispersers and their abundance and behavior. Pollinators and seed dispersers are undescribed for *A. woodmaniorum*. Phase 2 of the project aims to describe the mating system of *A. woodmaniorum* in terms of the level of outcrossing or selfing and degree of correlated paternity within pods, as well as investigating levels and patterns of gene flow via pollen for populations across the species range. These processes will be investigated in small populations with different degrees of isolation, as these factors are expected to impact on the mating system and patterns of gene flow.

Materials and Methods

Mating System

Seed pods from the 2009 flowering season were collected in December 2009. Pods were collected from one site in the main range (MA2) and from nine small isolated populations (see Figure 8). A total of 721 seed were processed, germinated in growth cabinets and grown in shadehouse conditions 9). Population size (the number of individuals) and degree of isolation (linear distance to individuals in the next closest population) for the populations are provided in Table 8.

Seed set and predation

A total of 718 seed pods from across the ten populations were assessed for seed production. A degree of seed predation was evident. We scored pods for the number of ovules (a sum of the number of viable seed and aborted seed observed within pods), the number of viable (fully developed) seed, the number of aborted (shriveled and undeveloped) seed, the number of seed predated by *Coleoptera* (beetle) larvae and the number of seed predated by mammals or birds (seed clearly chewed out of the pod). Values were converted to population totals and average percentages. Regression analysis was conducted to test whether the number of ovules, the number of viable seed or the number of aborted seed was correlated to population size or degree of isolation.

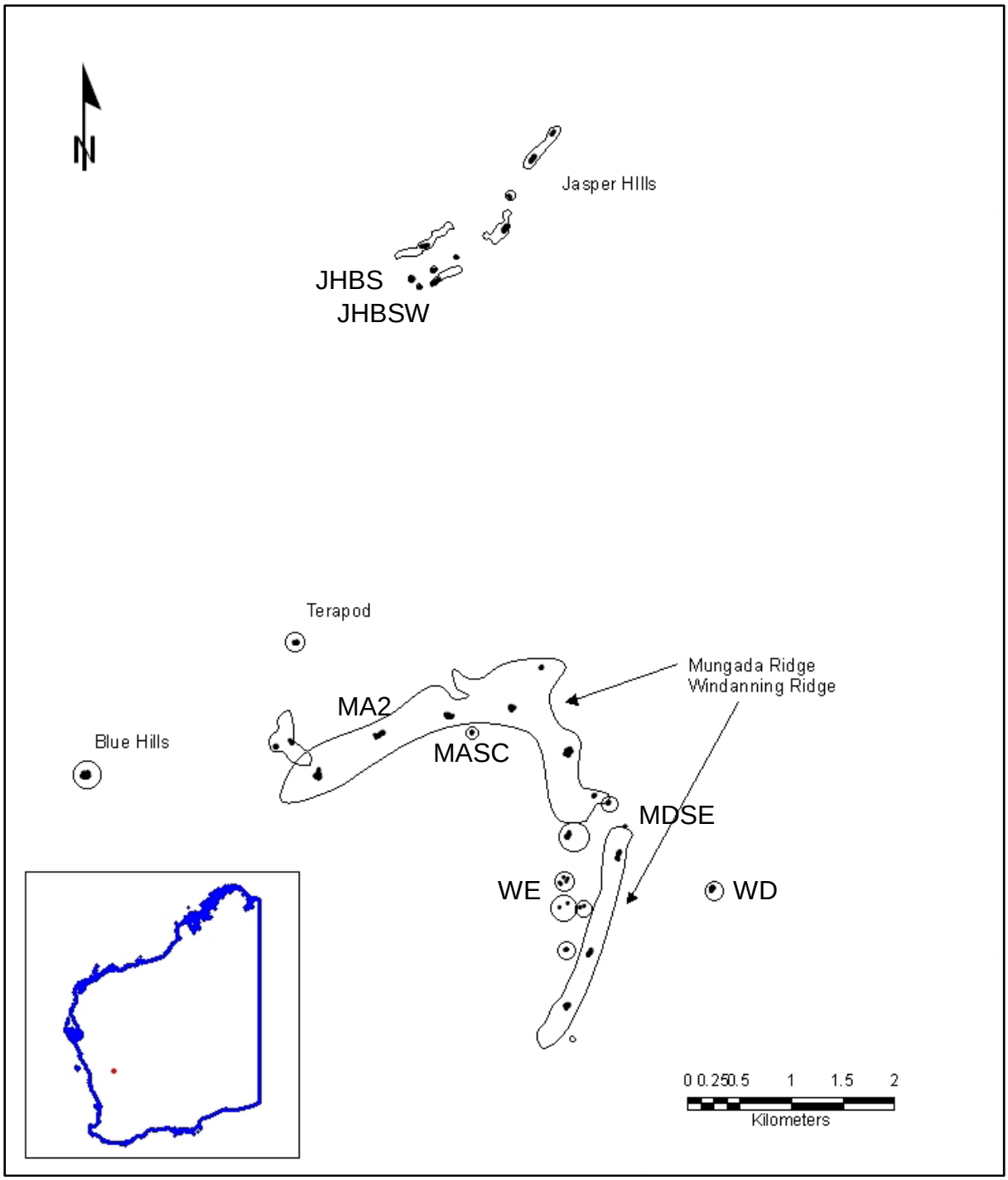

Figure 8. Range of *Acacia woodmaniorum*(open shapes) showing one correlated paternity site within the main range (MA2) and eight small gene flow sites (JHBS, JHBSW, MASC, MDSE, WD, WE, Terapod and Blue Hills) where pods were collected (dark circles). Regions are labeled.

Correlated Paternity within pods

A total of 139 seed representing all seeds within each of two pods from each of ten mother plants at the MA2 sampling site within the main range were sown for a pilot study assessing correlated paternity within pods. The site represents a subpopulation within the large continuous population occurring across Mungada Ridge where the effects of small population processes are expected to be minimised

i.e. pollinator abundance should not be reduced and outcrossing rates are expected to be high. In the *Acacia* genus pollen occurs as composite units or polyads with several grains, or as pollinia with very large numbers of grains. The pollen grain number usually exceeds the ovule number slightly so that a single polyad pollinating a stigma can fertilise all ovules within a flower (Kenrick& Knox 1982). In this case, all seed within a pod will be full sibs with the same father and correlated paternity within a pod will be high. It is important to assess the degree of correlated paternity in order to design studies of gene flow via pollen that are not biased by a large number of full sibs. 122 of the progeny survived and were harvested for DNA extraction and genotyped at nine microsatellite loci. As maternal genotypes are already known, genotyping of progeny allows the paternal (pollen) contribution to the progeny to be determined. Evidence for multiple paternities within pods can be tested in a number of ways.

1. Using a pollen array method. Using information from the total progeny array from each pod we determined the possible pollen genotype for loci where the maternal parent was homozygous. Progeny arrays were screened to detect more than two alleles in the pollen pool of a single pod which is unambiguous proof of multiple paternities.
2. Using the sibling pair method utilised in the MLTR program (Ritland 2002; Ritland& Jain 1981). This method uses maximum likelihood methods to determine the correlation of paternal outcrossed gametes or the proportion of full sib progeny among a pair of siblings (r_p). We calculated all mating system parameters simultaneously using the Newton-Raphson algorithm and the standard error obtained with 100 bootstraps. We determined the effective number of pollen donors per pod as $N_e = 1/r_p$.

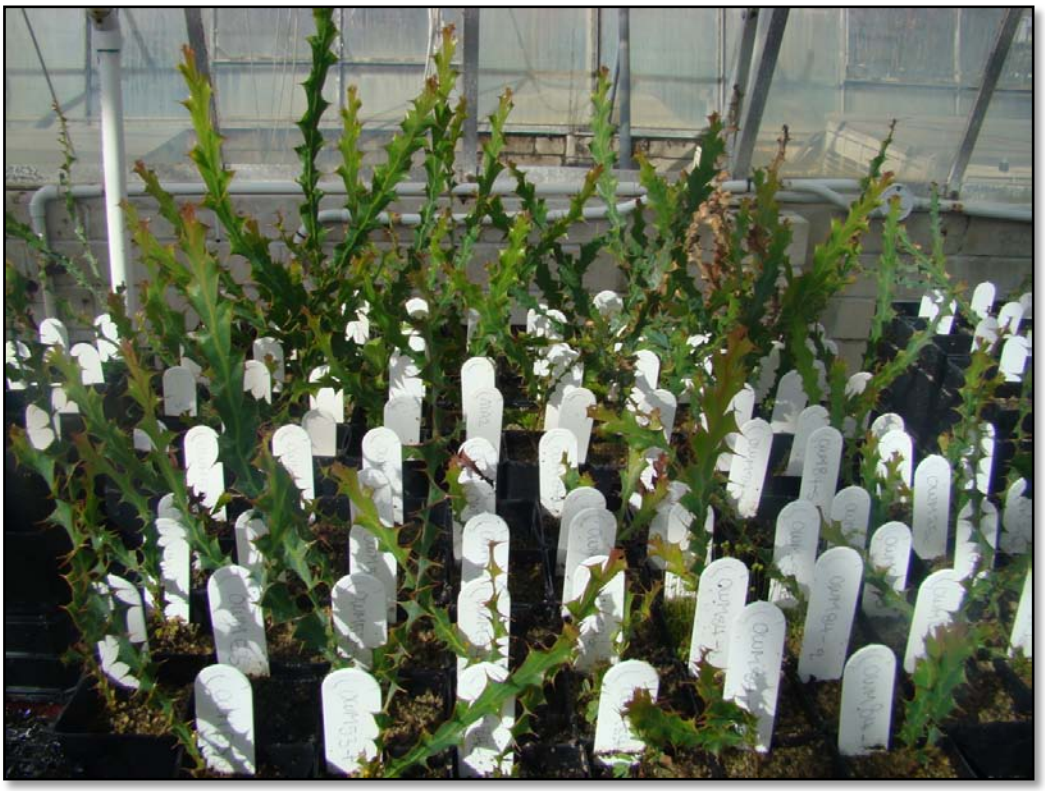

Figure 9. *A. woodmaniorum* progeny being grown for germplasm. Photograph by M.A. Millar.

Outcrossing Rate

A further 582 seeds representing progeny arrays of up to 22 seed from mother plants in eight populations were sown for further assessment of the mating system and patterns of pollen flow (Figure 9). Only a single seed was taken from a given pod so that results would not be biased by correlated paternity between seeds within pods. Mating system estimates could not be obtained using progeny from the single individual at WS which was excluded from analysis. A total of 408 progeny survived to be harvested for DNA extraction and genotyped at the same nine microsatellite loci. Mating system parameters were obtained for the eight populations' using the MLTR software (Ritland& Jain 1981). Estimates of the multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), the apparent level of selfing due to biparental inbreeding (t_m - t_s) and the multilocus correlated paternity among pods (r_p) were obtained for each population. Regression analysis was conducted to test for correlation between outcrossing rates and population size or degree of isolation.

Gene Flow

The same genotypic data from the 408 seedlings above was used for direct paternity analysis which allows more detailed investigation of patterns of pollen dispersal within and among populations than do indirect estimates based on F_{ST} values. For each of the eight populations, genotypic data for progeny arrays were analysed in conjunction with genotypic data for all adult trees (available from Phase 1 of the project). Direct paternity analysis was conducted using two programs and results compared. NEWPATXL is a paternity program that calculates allele frequencies, checks for the presence of null alleles and uses a randomization approach to assess the significance of matches found between parent offspring relationships (Amos available online at http://www.zoo.cam.ac.uk/zoostaff/amos/newpat.htm). A single set of allele frequencies were used for female plants/progeny, and potential male parents (as these are the same as the female plants) and potential fathers generated for each progeny. The program searches for matches between a progeny genotype and a potential father genotype and when a match is found the program simulates large numbers of progeny and males to determine how frequently such a match will arise by chance. The randomisation matches must have an equal or greater relatedness score compared with the match being tested so the number of matches in the randomisation tests represents the percent probability that a similarly sized data set will yield a match by chance. In this way the confidence of parentage assignments can be statistically evaluated. The program considers unscored loci and allows for mismatches or scoring errors. We ran the program for each population's data set allowing for 1 mismatch due to null allele between progeny and potential fathers. We let the program calculate the frequency of null alleles present at each locus in each population and used those values in calculations. For each progeny we obtained a most likely father and a randomisation score, however, taking a conservative approach, we calculated the pollen immigration rate as the percentage of progeny that could not be assigned any father from within the stand regardless of the randomization score.

The CERVUS version 3.0.3 program (Marshall *et al.* 1998) also finds optimal progeny/ father pairs but uses a maximum likelihood method of paternity analysis. CERVUS calculates allele frequencies using a method akin to NEWPATXL then uses simulation to estimate the resolving power of the set of loci. Simulation is used to calculate the

likelihood of parentage of the true parent and unrelated parents for a large number of progeny. In this way critical values of log-likelihood statistics can be obtained and the confidence of the real parentage assignments can be statistically evaluated. We ran the program for each populations data set with simulation of 10000 progeny, the known number of candidate fathers (total number of adult trees in the population), 100% of known candidate fathers genotyped, an error rate of 1%, and a minimum of 4 loci typed to obtain delta scores at a strict 95% confidence level and a relaxed 80% confidence level for assignment of fathers given a known mother. The program calculates the frequency of null alleles present at each locus in each population. We ran the program for each population allowing for one mismatch due to a null allele between progeny and potential father. As the outcrossing rate of *A. woodmaniorum* is close to 1.0 we did not allow known mothers to be potential fathers. For each progeny we obtained a single most likely father with 95% or greater confidence, between 95% and 80% confidence or less than 80% confidence, or we obtained a number of equally likely potential fathers within the population. Again, using a conservative approach we considered all most likely fathers assigned within a population as the most likely father, and calculated the immigration rate as the percentage of progeny that could not be assigned any father from within the stand.

We compared rates of pollen immigration (percentage of progeny within a population that could not be assigned a father within the population) obtained from the two methods. Regression analysis was conducted to test for correlation between pollen immigration rates and population size or degree of isolation. We used the results obtained from the CERVUS program for regression analysis as this program appeared to provide a more rigorous statistical analysis for selection of most likely fathers.

Results

Mating System

Seed set and predation

Seed set across the species range was low when as assessed by visual comparison with other *Acacia* species in the area for the same season. The number of ovules observed within mature pods ranged from 1 to 12 and averaged 6.48. An average of 6% of initially viable seed was predated by beetle larvae, which were in turn predated upon by a *Chalcidoid* wasp species. Seed predation was greatest at the main Mungada Ridge and Terapod areas. More than one seed was often predated within a single pod. Values of the number of pods examined, the average number of ovules, the percentage of viable seed, the percentage of aborted seed, the percentage of seed predated by beetle larvae and the percentage of seed predated by mammals or birds are provided for ten populations that were assessed for seed set and predation in Table 7. There was no significant correlation between the number of ovules, the number of viable seed or the number of aborted seed and population size or degree of isolation.

Correlated Paternity within pods

Progeny arrays within pods from population MA2 ranged in size from three to nine seed. Of nineteen pods examined one incidence of more than two paternal alleles was detected using the pollen array method. This leads to an estimated 93.33% of progeny within pods having a single father. The accuracy of this kind of analysis

depends on the level of polymorphism present at loci. In pods where only one or two paternal alleles were detected, all seed may have been sired by a single pollen grain; however if multiple pollen grains with similar genotypes fertilised a single stigma, all seed within a pod may still have identical genotypes. Pods where all seed have identical genotypes are therefore not definitive proof that multiple paternity has not occurred and estimates of correlated paternity may be biased upwards. Multilocus correlated paternity within pods calculated using the sibling pair method in MLTR was high, r_p = 0.492 (se = 0.097) and indicates an effective number of pollen donors per pod of 2.03.

Table 7. The number of pods examined, the average number of ovules, the percentage of viable seed, the percentage of aborted seed, the percentage of seed predated by beetle larvae and the percentage of seed that appeared to be predated by mammals or birds for ten populations of *A. woodmaniorum*.

Population	Number of pods examined	Average number of ovules	Percentage of viable seed	Percentage of aborted seed	Percentage of seed predated by beetle larvae	Percentage of seed predated by mammals or birds
JHBS	64	5.17	60	31	7	2
JHBSW	45	6.69	72	24	4	0
MA2	99	6.80	62	18	18	2
MASC	80	5.68	48	42	8	1
MDSE	88	7.03	81	17	1	0
Terapod	47	6.77	66	20	10	3
Blue Hills	132	6.67	79	18	2	1
WD	66	7.68	83	13	3	1
WE	91	6.81	66	29	3	1
WS	6	5.50	67	30	3	0
Total or Average	718	6.48	68	24	6	1

Outcrossing Rate

All estimates of multilocus outcrossing rates were high, averaging t_m = 0.98 for eight populations of *A. woodmaniorum* (Table 8). Single locus estimates were similarly high resulting in very low estimates for the degree of biparental inbreeding within populations (t_m-t_s). Levels of correlated paternity among pods produced low to negative values in all populations except Blue Hills (Table 8). There was no significant correlation between outcrossing rates or the degree of biparental inbreeding and population size or degree of isolation for these populations.

Gene Flow

Rates of pollen immigration into populations (i.e. the percentage of progeny for which no likely candidate father can be assigned within the population and which must there for be fathered by pollen entering the population from another population) varied but were considerable for all populations studied. Immigrant pollen was attributed to a mean of 45% and 40% of all mating with the two programs (Table 9). Rates of pollen immigration obtained from analysis with NEWPATXL were not always similar to those obtained with CERVUS. Further details of the paternity analysis for progeny of the eight populations obtained via CERVUS are provided in Table 10. Estimated levels of pollen immigration into populations varied from 13% to 61% and

averaged 40% for eight small isolated populations of *A. woodmaniorum*. Note; there is a small discrepancy in the final average values in Tables 9 and Tables 10 due to rounding of values.

Table 8. Population size, isolation distance, and estimates of the multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), the apparent level of selfing due to biparental inbreeding (t_m - t_s) and the correlated paternity among pods (r_p) for eight populations of *A. woodmaniorum*.

Population	Size	Isolation (m)	t_m	t_s	t_m-t_s	r_p
JHBS	22	120	0.91	0.92	-0.01	0.02
JHBSW	17	120	1.00	0.99	0.01	0.00
MASC	8	200	1.00	0.99	0.01	0.05
MDSE	7	200	1.00	0.94	0.06	0.10
Terapod	10	700	1.00	0.99	0.01	0.00
Blue Hills	146	1870	0.99	0.97	0.02	0.18
WD	29	970	0.90	0.90	0.00	0.10
WE	16	180	1.00	1.00	0.00	0.04
Average			0.98	0.96	0.01	0.06

Table. 9. Rates of pollen immigration obtained from analysis with NEWPATXL and obtained with CERVUS for 403 progeny in eight populations of *A. woodmaniorum*. Values are percentages of progeny analysed.

		Program			
		NEWPATXL		CERVUS	
Population	Number of progeny assessed	Within population	Pollen immigration	Within population	Pollen immigration
JHBS	28	79	21	82	18
JHBSW	18	50	50	39	61
MASC	49	58	42	44	56
MDSE	70	46	54	44	56
Terapod	25	25	75	46	54
Blue Hills	103	64	36	87	13
WD	40	60	30	78	23
WE	70	51	49	59	41
Total or Average	403	55	45	60	40

For four populations (MASC, MDSE, WE and T) there was a high level of discrimination among potential fathers within the population. For the other four populations there was a degree of ambiguity regarding the most likely father (i.e. a high proportion of progeny assigned a most likely father within the stand at a confidence level < 80%. Percentage of pollen immigration is illustrated for the eight small populations in Figure 10.

There was no significant correlation between pollen immigration rates and the size of populations (R^2 = 0.4427, p = 0.0717) or degree of population isolation (R^2 = 0.0533, p = 0.6184).

Table 10 Categories into which progeny from eight populations of *A. woodmaniorum* fall when analysed for paternity using CERVUS. Categories include 95%; a most likely father is found within the population at a confidence level of 95% or greater, 80%; a most likely father is found within the population at a confidence level of 80% or greater, >80%; the sum of the first two categories, <80%, a most likely father is found within the population at a confidence level less than 80%, More than one father; a single most likely father cannot be found within the population i.e. multiple fathers have equal likelihood of being the real father, Immigration; no father can be assigned within the population so progeny must be a result of immigrant pollen. Values are provided as percentages of progeny.

	Most likely father within population					Most likely father outside population
Population	95%	80% - 95%	>80%	<80%	More than one most likely father	Immigration
JHBS	25	32	57	14	11	18
JHBSW	6	6	11	6	22	61
MASC	38	6	44	0	0	56
MDSE	29	16	44	0	0	56
T	21	25	46	0	0	54
Blue Hills	8	26	33	49	5	13
WD	25	30	55	23	0	23
WE	30	27	57	1	0	41
Average	23	21	43	12	5	40

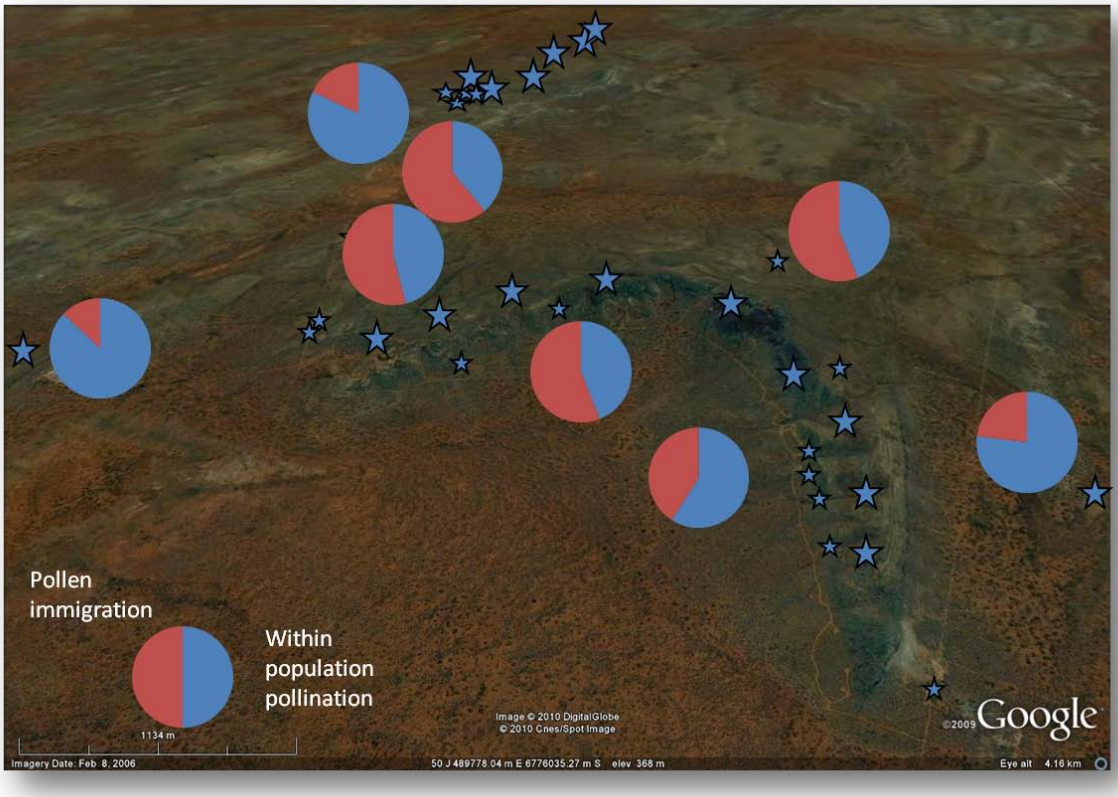

Figure 10 Percentage of progeny resulting from pollen immigration into eight small isolated populations of *A. woodmaniorum* (red). Background image courtesy of Google earth.

Discussion

Mating System

Observations suggest that *Acacia woodmaniorum* has a low level of pod set and seed maturation across its range in comparison with many other sympatric *Acacia* species. Observed predation by mammals and or birds was low across all populations but values do not include instances where entire pods have been removed, which may significantly impact on the number of mature pods observed and seed available for recruitment. All populations where seed collections were made showed evidence of seed predation by *Bruchidius* sp. (seed beetle) beetle or *Malanterius* sp. (weevil) larvae. The Bruchidea family is found worldwide although indigenous Australian species are poorly described. It is more likely the beetles are *Malanterius* sp. which are abundant in Australia and host specific to *Acacia* (New 1983). These small beetles pierce developing seed pods with their sharp ovipositors and lay their eggs within, next to or on a ripening seed. The hatched larvae then bore into the developing seed on which it feeds. When fully grown the larvae leaves the seed which has typically dehisced and the larvae pupates in the soil before emerging as a beetle. Because of their complex life cycle no adult beetles were observed so accurate species identification could not be made. The proportion of affected seed was variable among populations, ranging from 1% to 18% of presumably initially viable seed. Predation was greatest in the populations from the central region of the species range. The degree of weevil attack (average of 6%) was only slightly lower than that observed by New (1983) for 11 *Acacia* species (average of 9% and 8% of seed over two seasons) although their data is heavily biased by a severe attack in one species one season. It is difficult to estimate what effect predation by beetles, birds, or mammals has on recruitment levels in *A. woodmaniorum*. It is recommended that predatory beetles be collected for identification and observations of predatory birds and mammals be conducted if future studies of pollinators and seed dispersers are conducted.

Neither the number of ovules nor the number of pollen grains in the polyad has been described for *Acacia woodmaniorum*. The maximum number of seed or fertilised ovules containing aborted seed observed in 718 mature pods was 12, which is evidence that the number of ovules in this taxon is 12 or greater. Kenrick and Knox (1982) suggest that the most common number of pollen grains in the polyad for the *Acacia* genus is 16 and that the pollen grain number usually exceeds the ovule number slightly, so that a single polyad pollinating a stigma can fertilise all ovules within a flower. In *Acacia woodmaniorum* an average of only 6.5 fertilised ovules (containing either aborted or mature seed) were observed in mature pods and this number was often much lower. This result suggests that, if the *A. woodmaniorum* polyad contained 16 pollen grains, a proportion of these, that are capable of successfully fertilising one of the 12 or more ovules do not do so. An alternative explanation is that polyads of *A. woodmaniorum* are comprised of less than 16 or 12 polyads, more than one polyad often fertilises flowers and only pollen grains from a compatible polyad (outcrossed or otherwise less related) are successful at fertilisation. There was no significant correlation between the average number of ovules and population size or degree of isolation.

Estimates of correlated paternity within pods obtained with the sibling pair method were slightly lower than expected given the frequency of the genotypes of all seed within pods being compatible with a single paternity. The statistical procedure utilised in MLTR determines the degree of relatedness between parents and each progeny and then compares this for pairs of sibs (seeds) to determine the correlation of paternity for the pair and provides an overall value for all seed within all pods. Values of r_p using Ritlands method are known to be underestimates (Hardy *et al.* 2004) and an estimate of rp = 0.492 obtained may be biased downwards. Estimates of correlated paternity among pods in the eight small populations of *A. woodmaniorum* were generally low suggesting large effective population sizes are maintained even in small isolated populations. Low correlated paternity among pods suggests that pollination among different flowers on a maternal plant is affected by highly unrelated pollen grains, as would be expected with a highly outcrossed mating system and the existence of a self incompatibility mechanism. Low correlated paternity among pods is evidence that mate availability is not limited by a low number of pollen donors, as may be expected in small populations. Low levels of correlated paternity among pods also indicates that co-dispersion of pollen from the same source, for example when a pollinator visits nearby flowers and a pollen load from a single source pollinates several flowers, is limited in *A. woodmaniorum*. A high degree of co-dispersion would be expected in species in which there is significant heterogeneity among flowering intensity, density and or timing and the result supports field observations that suggest a relatively short homogeneous flowering period in *A. woodmaniorum*.

We found evidence of seed abortion in 13% to 42% of initially fertilised ovaries observed within pods of *A. woodmaniorum*. These un-developed seed showed no sign of beetle attack. The maximum and average values of 24% of seed aborted are greater than that observed for four eastern states *Acacia* species (an average of 10%, New 1983). In general, reasons for post fertilisation seed abortion are unknown. Seed abortion could be a result of severe environmental pressures, such as a lack of water available for developing seeds. Preferential resource allocation based on the position of the seed in the pod, predation or pathogen infestation or paternal fitness has been evoked to explain why a percentage of embryos or seeds abort (Bawa& Buckely 1989; Tybirk 1993, 2007). Alternatively preferential seed abortion may be due to the effects of a post zygotic lethal system where inbred or selfed seed is preferentially aborted due to the action of post zygotic lethal (Kenrick *et al.* 1986; Morgan *et al.* 2002). There was no significant correlation between the number of viable seed or the number of aborted seed, and population size or degree of isolation. These results are all congruent with the maintenance of a highly outcrossed seed crop via high levels of gene flow, with a degree of self incompatibility that results in preferential retention of more outcrossed seed and abortion of selfed or otherwise more inbred seed.

Gene Flow

Pollen immigration into small and or isolated populations of *A. woodmaniorum* varied but the average level for eight populations was high, with a conservative estimate of immigrant pollen for 40% of all matings (CERVUS results). There was a trend of increased pollen immigration into smaller populations as may be expected due to

variable rates of pollen production when small populations are located near larger populations. There was also a trend of decreased pollen immigration into more isolated populations as expected when pollinator behaviour follows a pattern of a decreasing number of trips among populations with increasing geographic distance or when pollen dispersal by wind becomes limited with distance. Neither of these trends were statistically significant however, which further suggests little impediment to pollen immigration into small or isolated populations of *A. woodmaniorum.* The finding of high levels of pollen immigration via direct paternity analysis is congruent with the low level of genetic differentiation evident among populations and the high number of migrants among populations as estimated via F statistics of *A. woodmaniorum.*

Pollen immigration events were detected over large distances, of up to 1870 m, in *A. woodmaniorum*. Dispersal may occur over greater distances with pollen travelling into populations from those other than the next closest population. Most likely fathers from outside the population cannot be assigned with any confidence using direct paternity methods however due to the number of potential fathers across the species range. We have used a conservative approach that assumes pollen immigration is from the next nearest population.

Conclusion

Seed set in *A. woodmaniorum* is low when compared to other Acacias in the area. Some seed is predated by beetles and birds or mammals and predation by beetles is significant. The level of seed abortion is high and is likely linked to a degree of self incompatibility. A more detailed study of the reproductive biology of *A. woodmaniorum* including scanning electron microscopy of the pollen grain, conducting controlled crosses and microscopy of pollen tube growth and embryo development, could aid in further elucidating the nature of the mating system, including any pre-zygotic or post-zygotic incompatibility mechanisms. The maintenance of high outcrossing rates in small and isolated populations is further evidence for a degree of self incompatibility in this species. High levels of pollen immigration into small and isolated populations indicate that either insect pollinator visitation to these populations occurs frequently, or alternatively, if the species is wind pollinated, that wind pollination is effective across the species range. Detailed studies of animal or insect pollen dispersers, as well as seed dispersers, are suggested as their conservation will assist in the future maintenance of large effective population sizes, levels of genetic diversity and outcrossing rates in small isolated populations of *A. woodmaniorum*.

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