Chapter 5 *Macadamia integrifolia* Mating and Breeding Systems

5.1 Introduction

The mating system of a population or species can be defined as the genetic relatedness and patterns of pairings between gametes (Ward et al. 2005); and the reduction in size and increased isolation of populations frequently brought about by habitat fragmentation is predicted by population genetic theory to have direct implications for plant mating systems, and consequently, population viability (Buza et al. 2000). These may include lack of mates, pollinator limitation, reduced reproductive success and fitness through increased levels of inbreeding (Buza et al. 2000; Hall et al. 1994; Krauss 2000; Young & Brown 1999), smaller effective population size and loss of genetic diversity due to reproductive dominance of a small subset of trees (Aldrich & Hamrick 1998). The severity of these implications of mating system change is often informed by a species' breeding system, and self-incompatible species with highly specialised plant-pollinator relationships are often the most affected (Didham et al. 1996). In the long-term, altered patterns of mating may consequently result in genetic decline and population extinction (Frankham 1996).

Breeding systems may be defined as the morphological and physiological characteristics of pairing (Neal & Anderson 2005). In orchard studies, *M. integrifolia* has been observed to possess a partial gametophytic self-incompatibility system (Ito et al. 1983; Sedgely 1983). The predominant pollinators are native bees of the genus *Trigona* and the introduced honeybee, *Apis mellifera* (Heard et al. 1987; Wallace et

al. 1996), however little is known of the mating system of the species in the wild. Out-crossing experiments in orchards observed varying compatibility between different cultivar combinations, but no relationship has been observed between nut set and parental genetic distance (Vithanage et al. 1998). With the potential importance of mating and breeding system for population viability, this chapter aims to: (i) estimate the impact of fragmentation on the mating system of *M. integrifolia*; and (ii) determine the breeding system of *M. integrifolia* in the wild.

5.2 Methods

5.2.1 Mating System Analysis

While mating system patterns as deduced though progeny arrays and paternity analysis allow insight into the contemporary mating system of a population, changes in mating system typically require multiple generations to become apparent in the genetics of the adult population. Through assessment of both population genetic indicators of mating system and contemporary gene flow, investigation of both past (historic) and present (contemporary) mating systems is therefore possible and has been undertaken in this study.

5.2.1.1 Historic Mating System

All reproductive plants and a sample of greater than fifteen seedlings and juveniles (defined as plants under two metres in height) where available in each study site were genotyped (600 adults and 409 juveniles total). Individuals were genotyped for two microsatellite loci (*STMS1* and *STMS11*) developed by Vithanage et al. (1999) and

three RAMiFi loci (*B11.043mi*, *B11.072mi* and *A06.100mi*) described by Peace et al. (2004) using the same protocol as for the RAF markers (Waldron et al. 2002). The RAMiFi locus B11.043mi was not included in the analysis due to its high mistyping rate of 0.20.

Population inbreeding coefficients (F_{IS}) were calculated using the program Genepop on the Web (Raymond & Rousset 1995). Due to the relatively short period of time since habitat fragmentation is estimated to have first taken place for the small and medium study sites, it is possible that many of the remaining adult trees may be remnants from the pre-fragmentation population. Differences in F_{IS} values between adult and juvenile cohorts were therefore also investigated. Adults in this study were defined as trees greater than two metres in height, based on the observation that plants under two metres were rarely observed to flower. Plants smaller than two metres were classified as juveniles. A two-way ANOVA was used to test for significant differences between site classes, cohorts, and the interaction between the two.

5.2.1.2 Contemporary Mating System

Contemporary mating system and fine-scale patterns of gene flow were investigated in two adjacent sites in the Samford Valley. The two sites, sites 20 and 77, were separated by 2.8 km of rural land and were both of medium size. Searches of the region revealed no additional *M. integrifolia* individuals. Remnant forest in site 77 was substantially less contiguous than in site 20, with most forest cover limited to creek banks.

In 2003, ten to 15 nuts were collected from throughout the canopy of each of 11 trees across both sites (153 nuts total) and were genotyped along with all adult plants observed to flower in the two sites in 2003 (189 trees). Almost all maternal trees were located on the edge of the fragments or out in the open, as very few trees within fragments produced adequate nuts for this study. This may have implications for the results as the selected maternal trees may be more attractive to pollinators due to their position and large numbers of racemes.

Individuals were typed for seven microsatellite loci (STMS1, STMS11, MinuS1a, MinuS5, MinuS7, MinuS14, MinuS74; Schmidt et al. 2006; Vithanage et al. 1999) and three RAMiFi loci (B11.043mi, B11.072mi, A06.100mi; Peace et al. 2004). RAMiFi locus B11.043mi was removed from the data for both analyses, however, due to its high mistyping rate. The presence of null alleles in loci with a frequency greater than 0.15 was dealt with in a conservative manner, and all apparent homozygotes in sites with null frequencies over this amount were treated as heterozygotes with a null allele. The presence of null alleles would therefore have been unlikely to result in a false paternal exclusion.

5.2.1.2.1 Mating System Parameters

The contemporary mating system of *M. integrifolia* was discerned primarily using the estimation procedures of Ritland (2002), based on the mixed mating model of Brown and Allard (1970). Using maximum likelihood procedures, Ritland's MLTR software was employed to calculate multilocus outcrossing rate (t_m) using the Newton Raphson method and average single locus outcrossing rate (t_s). Patterns of paternity, which

affect local patterns of genetic variation and the effectiveness of local selection (Sun & Ritland 1998), were also measured with parameters of the 'correlated mating model' of Ritland (1989). Correlation of outcrossed paternity within progeny arrays, or probability that a randomly chosen pair of progeny from the same array are full sibs (r_p) was estimated using the Newton Raphson method (Ritland 1989), as well as correlations of selfing among loci (r_s), which does not suffer bias due to the number of loci analysed, unlike (t_m - t_s), which similarly provides an estimate of the apparent selfing due to biparental inbreeding (Ritland 1989, 2002). Variances of the above quantities were calculated using the bootstrap method where the progeny array (within families) is the unit of resampling (1000 bootstrap replicates).

5.2.1.2.2 Paternity Analysis

Paternity analysis was also conducted in sites 20 and 77 to trace contemporary pollen movement contributing to successful fertilisation. Because paternity allocation by complete exclusion techniques was not possible due to potential false exclusions from genotyping errors and inadequate distinction between individual genotypes for full exclusion, paternity was assigned using the categorical allocation method of the program Cervus (Marshall et al. 1998). This technique assigns progeny to non-excluded parents based on likelihood scores derived from their genotypes and is currently the most common approach to paternity analysis (Jones & Ardren 2003).

Pair-wise relatedness (r) (Lynch & Ritland 1999) and spatial distances between the identified parents were also estimated using the software SPAGeDi (Hardy & Vekemans 2002), allowing analysis of patterns of relatedness and spatial distances

associated with gene flow. Statistical analysis of relatedness and spatial distance was conducted using one-way ANOVA. Differences in F_{IS} between parents and offspring in sites 20 and 77 were calculated using a *G*-test with Williams (1976) correction factor.

In 2003, flowering synchronicity was analysed at sites 20 and 77 to estimate for each reproductive tree the probability of fertilising the maternal trees, based on the proportion of the total site pollen pool contributed by each tree relative to the flowering period of each maternal tree. The number of fully open racemes was counted on all trees in the study sites once a week from the beginning of the flowering period. Weekly intervals were selected as racemes of *M. integrifolia* remain open for just under this length of time (Heard 1993), therefore ensuring that each raceme was counted once only. After seven weeks the number of new racemes being produced was extremely low and the census was discontinued. Flowering synchronicity between individual *i* and maternal tree *m* was calculated as:

$$S_{i,m} = \sum_{1}^{t} \left(\frac{n_{i,t}}{N_t} \times \frac{n_{m,t}}{n_m} \right)$$

where $n_{i,t}$ is the number of open flowers on individual *i* at time *t*, N_t is the total number of open flowers in the site at time *t*, $n_{m,t}$ is the number of open flowers on the maternal tree at time *t* and n_m is the total number of open flowers observed on the maternal tree. Note that while estimation of flowering synchronicity only takes into account trees from the same site as the maternal tree, pollen flow into the site from outside will only alter the magnitude of synchronicity, not the relative values themselves.

5.2.2 Breeding System Analysis

To investigate the breeding system of *M. integrifolia* in the wild, controlled crosses were performed at sites 20 and 77 in 2002 and 2003. Five trees were selected at each of sites 20 and 77 (both medium sites) to be pollen recipients (herein referred to as maternal trees) based on availability and accessibility of racemes. For each maternal tree, ten to 20 racemes per treatment were pollinated where possible, and for every treatment raceme an additional raceme was tagged and left unbagged for open pollination.

Measures of both genetic and spatial distance were utilised in selecting crossing partners, and the following crosses were carried out: (i) selfing (coded 'S'); (ii) genetically Similar trees Within the same population (WS); (iii) genetically Dissimilar trees Within the same population (WD); (iv) genetically Similar trees from Local populations (3-4 km away) (LS); (v) genetically Dissimilar trees from Local populations (LD); and (vi) genetically Dissimilar trees from Distant populations (100-150 km) (DD). Open pollinated controls were also included as a background comparison of natural fertilisation success. The crosses performed are summarised in Table 5.1 below.

In order to select appropriate pollen donors, genetic distances between maternal trees and all other reproductive plants in the two sites were estimated using dominant RAF markers (Waldron et al. 2002), as codominant markers were not available at the time. All samples were amplified using the primer B11, generating 44 dominant, polymorphic markers. Tanimoto genetic distance (Deichsel & Trampisch 1985), an

index equivalent to the Jaccard index (Salguiro et al 2004), was calculated between all flowering individuals using the program Spatial Genetic Software (Degen et al. 2001b). Tree pairs were classified as similar if they shared more alleles than would be expected by chance from a random sample of the population and dissimilar individuals shared fewer alleles than expected by random, based on thresholds of significant genetic similarity and dissimilarity from plots of spatial genetic distance classes against mean genetic distance (distograms) generated by Spatial Genetic Software. Due to the low number of dominant markers available for this analysis at the time of cross-pollination, relatedness between parent pairs was confirmed at a later date using *r* values (Queller & Goodnight 1989) generated from the nine microsatellite markers run for paternity analysis.

Donor and recipient racemes were sealed inside paper bags at one day pre-anthesis, preventing contact by pollinators and therefore unwanted pollen deposition (Figure 5.1). The cross-pollination technique used was based on that of Ito and Hamilton (1969). Receptor racemes were randomly located throughout the accessible canopy area of each maternal tree (up to approximately five metres above ground height), avoiding racemes with fewer than 50 florets to avoid compensation for small floret numbers in final fruit set (Trueman & Turnbull 1994). After anthesis, pollen was collected from donor racemes using glass test tubes (internal diameter = 2.5 cm, length = 25 cm) containing silica gel and stored at approximately 4 °C until cross-pollination. Pollen was stored for up to a maximum of two days in this manner before crossing (Figure 5.2). Initial fruit set (IFS) was determined at 14 days and 28 days post-pollination, and final fruit set (FFS) ascertained five months later in late February/early March. FFS was included in this study following the findings of

Wallace et al. (1996) where IFS, used as an indicator of cross compatibility, was unreliable, with large variance in results between IFS and FFS and between years.

The effects of developmental stage (IFS1, IFS2 and FFS), cross and year on the number of nuts produced per raceme were analysed using a mixed model residual maximum likelihood (REML) analysis following the protocol described in section 4.2.2.1 and including the random factor maternal tree + maternal tree × year + maternal tree × cross + maternal tree × cross × year.

Figure 5.1 Bagged donor and recipient racemes on tree 77-13 in site 77 (2002). Racemes were sealed inside paper bags at one day pre-anthesis to prevent unwanted pollen deposition.

Figure 5.2 Cross-pollination of a maternal tree in site 20 (2002), performed by inserting recipient racemes into a glass test tube internally coated with donor pollen and rotating to allow pollen deposition onto the pollen presenters.

Table 5.1 Summary of cross pollinations performed in 2002 and 2003. Maternal and paternal tree numbers = (study site-tree number). O = open pollinated; S = selfed; WS = genetically Similar pollen parent from Within the same population as the maternal tree; WD = Within-population, genetically Dissimilar; LS = Local population (3-4 km), genetically Similar; LD = Local population, genetically Dissimilar; DD = Distant population (>100 km), genetically Dissimilar.

Maternal tree no.	*Cross type*	*No. of racemes treated*	*Cross break-down: Number of racemes treated (paternal tree no.)*
20-157	O	58	58
	S	21	21 (20-157)
	WS	13	13 (20-170)
	LD	10	1 (77-1), 2 (77-13), 5 (77-40), 2 (77-41)
	DD	9	9 (134-21)
20-162	O	37	37
	S	10	10 (20-162)
	WS	10	4 (20-157), 6 (20-170)
	LD	10	10 (77-13)
	DD	10	1 (134-21), 6 (8-215), 2 (8-266), 1 (8-272)
20-170	O	47	47
	S	11	11 (20-170)
	WS	10	10 (20-98)
	LD	13	3, 1 (77-1), 1 (77-13), 5 (77-37), 3 (77-41)
	DD	11	5 (134-21), 4 (8-215), 2 (8-272)
77-13	O	25	25
	S	18	18 (77-13)
	WS	16	3 (77-11), 10 (77-14), 3 (77-18)
	WD	6	6 (77-90)
	LS	13	4 (20-170), 9 (20-25)
	LD	9	5 (20-156), 4 (20-170)
	DD	6	6 (70-5)
77-40	O	53	53
	S	12	12 (77-40)
	WS	6	5 (77-1), 1 (77-41)
	WD	9	9 (77-90)
	LD	20	7 (20-157), 7 (20-167), 3 (20-170), 3 (20-98)
	DD	17	12 (134-21), 1 (8-251), 4 (8-266)
77-41	O	17	17
	S	4	4 (77-41)
	WS	4	2 (77-25), 2 (77-40)
	WD	1	1 (77-90)
	LS	1	1 (20-162)
	LD	11	5 (20-156), 2 (20-157), 4 (20-162)
	DD	16	4 (134-21), 5 (7-1), 3 (8-2), 4 (8-266)
77-90	O	35	35
	S	28	28 (77-90)
	WS	16	15 (77-80), 1 (77-82)
	WD	11	2 (77-13), 3 (77-14), 2 (77-18), 3 (77-4), 1 (77-41)
	LD	26	13 (20-162), 13 (20-170)
	DD	28	9 (134-21), 10 (70-37), 8 (8-251), 1 (8-266)
Total	A	16	
	O	272	
	S	104	
	WS	75	
	WD	27	
	LS	14	
	LD	99	
	DD	97	

5.3 Results

5.3.1 Mating System

5.3.1.1 Historical Mating System

Mean F_{IS} across all sites and cohorts was -0.023, with no difference observed between site classes, cohorts, or the interaction between the two (Table 5.2).

Table 5.2 Results from an ANOVA of FIS across site classes (small, medium and large) and cohorts (seedling/juvenile and adult).

	d.f.	*m.s.*	*Chi pr*
Site class	2	0.007	0.334
Cohort	1	0.002	0.522
Site class x cohort	2	0.008	0.291

5.3.1.2 Contemporary Mating System

Mating system parameters are summarised in Table 5.3. Multilocus outcrossing rates (t_m) in both sites are high, although slightly lower in site 77 than 20, and 39% of outcrossed progeny in site 77 were seen to share the same father, compared to only 15% in site 20. True selfing was attributed to 1.9% and 4.2% of the crosses analysed for sites 20 and 77, respectively, and biparental breeding for 2.1% and 6.9%, respectively.

Table 5.3 Mating system parameters (and standard errors) for sites 20 and 77 estimated using MLTR (Ritland 1989, 2002). tm = multilocus outcrossing rate; ts = average single locus outcrossing rate; r_p = the correlation of outcrossed paternity within progeny arrays (fraction of siblings that shared the same father); rs = correlation of selfing among loci.

Site	t_m	t_s	r_p	r_s
20	0.959 (0.073)	0.916 (0.048)	0.152 (0.088)	0.483 (0.370)
77	0.889 (0.131)	0.702 (0.129)	0.390 (0.294)	0.375 (0.254)

Of the 153 offspring assessed for paternity analysis, 19 had fathers assigned with ≥ 95% confidence, 119 with 80-95% confidence, and paternity of the remaining 15 was unresolved. Among the 138 progeny for which fathers were assigned, seven (5%) were the apparent result of selfing, 16 (12%) had fathers identified from the neighbouring population (i.e. from site 20 for nuts produced in site 77 and vice-versa), and the remaining 115 (83%) were from other pollen donors within the site. Paternal diversity was relatively high, with 66 different fathers contributing to the pool of offspring.

Figure 5.3 shows the frequency of pair-wise relatedness for (i) mothers and all possible fathers, and (ii) mothers and assigned fathers. The latter group was found to possess slightly higher levels of relatedness ($F_{1,2096}$=33.48, P<0.001), with mean relatedness being -0.01 for mothers and all fathers and 0.13 for mothers and assigned fathers.

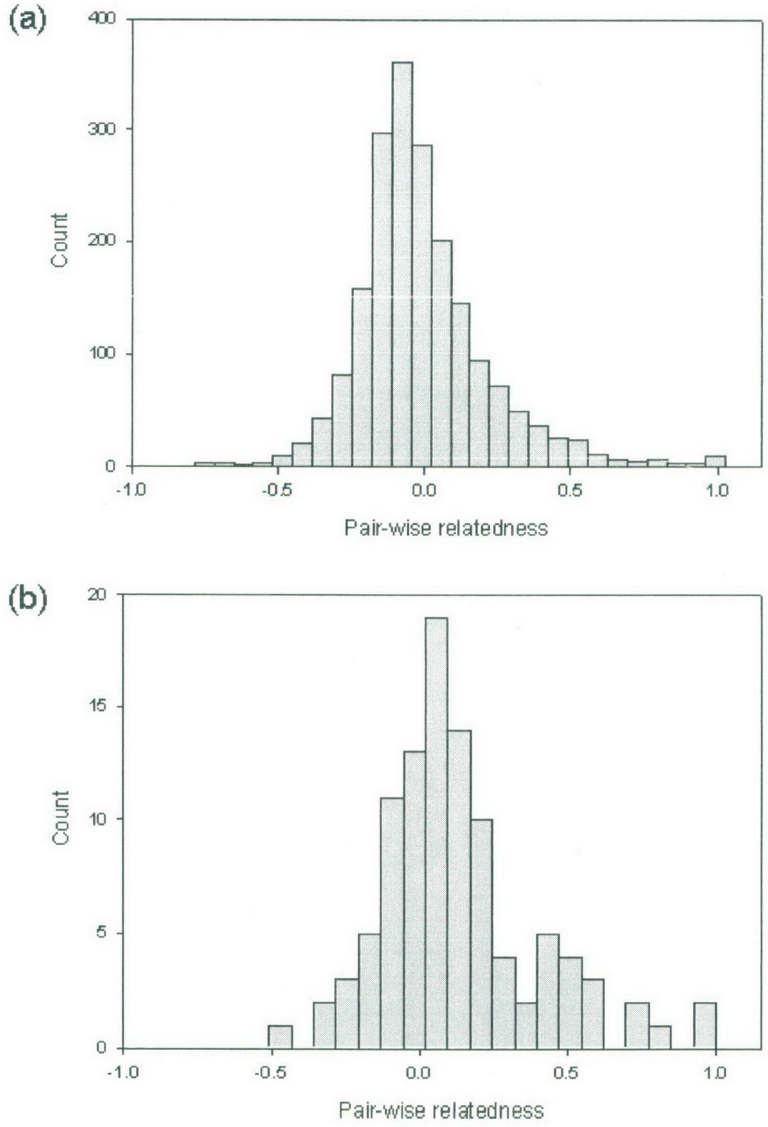

Figure 5.3 Histograms of pair-wise relatedness (Lynch & Ritland 1999) for: (a) mothers and all possible fathers in both sites, and (b) mothers and all assigned fathers.

Mean relatedness between mothers and assigned fathers within the same site was significantly greater in site 77 than between mothers and all possible fathers in the same site (Figure 5.4), with values of 0.36 and 0.07respectively. No significant difference was observed in site 20 between the two groups. Mean inter-tree distance was not significantly different between the two groups in site 77, however inter-tree distance was slightly lower between identified parents in site 20 compared to mothers and all possible fathers, with average distance of 205 m between mothers and

assigned fathers and 153 m between mothers and all possible fathers. Inter-site pollen flow averaged 2.8 km between parental trees. Flowering synchronicity with maternal trees was positively associated with paternity in site 20 but not in site 77 (Figure 5.4).

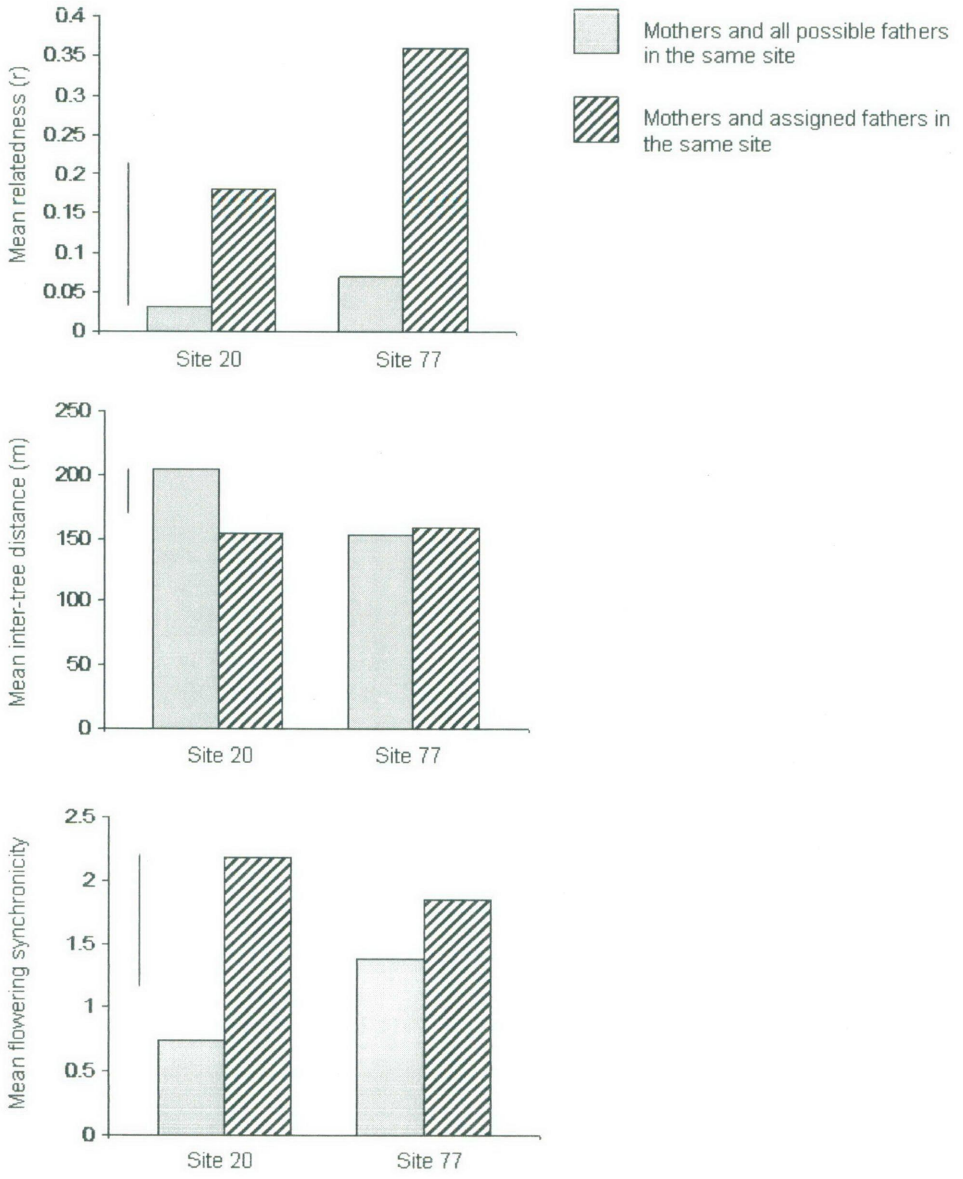

Figure 5.4 Mean relatedness, inter-tree distance and flowering synchronicity (+ LSD) between mothers and all possible fathers, and mothers and fathers assigned in the paternity analysis, for sites 20 and 77. Comparisons are of individuals within the same site.

F_{IS} values for the parents and open-pollinated progeny in sites 20 and 77 are presented in Table 5.4. Mean homozygosity levels were not significantly different between parents and offspring or sites (G=0.0102, P=0.100).

Table 5.4 F_{IS} values for the identified parents and open-pollinated progeny, calculated using Genepop on the Web (Raymond & Rousset 1995).

Site	*Group*	*F_{IS}*
20	Parents	0.041
	Progeny	0.001
77	Parents	0.019
	Progeny	0.059

5.3.2 Breeding System

In the analysis of pollination success per raceme, only stage (number of nuts per raceme at two, four and 25 weeks post-pollination) and year were significant at the P<0.05 level (Table 5.5). The mean number of nuts per raceme across all treatments declined significantly between IFS1 and FFS (Figure 5.5), and significantly more nuts were produced from crosses in 2003 compared with 2002 (effect = 0.579, s.e.d. = 0.247).

Table 5.5 Wald test results for the effect of stage, cross and year on number of nuts per raceme from the cross-pollination experiment. * = significant at P<0.01.

	d.f.	*Wald/d.f.*	*Chi pr*
Stage	2	258.21	<0.001*
Cross	7	0.81	0.582
Year	1	9.45	0.002*
Cross x stage	14	1.39	0.147
Cross x year	5	1.58	0.162

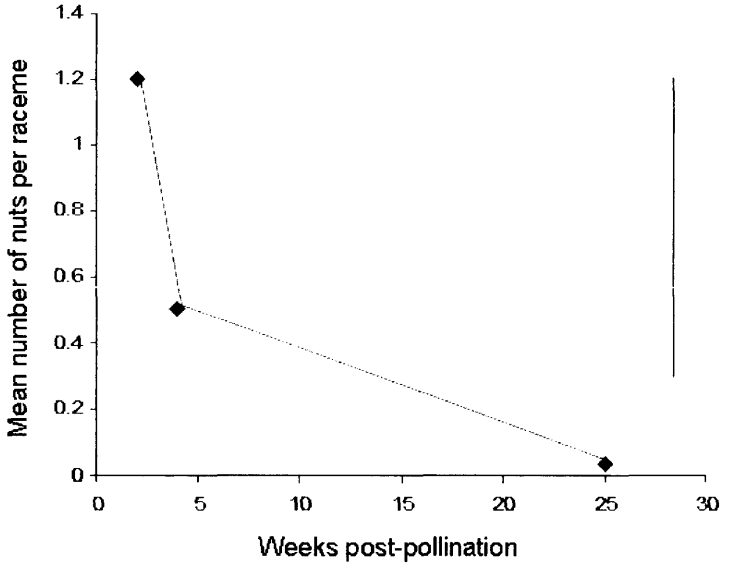

Figure 5.5 Corrected mean number of nuts per raceme across all treatments at IFS1 (two weeks post-pollination), IFS2 (four weeks post-pollination) and FFS (approximately 25 weeks post-pollination) +LSD.

5.4 Discussion

5.4.1 Mating System

F_{IS} values across site classes and cohorts indicate that *M. integrifolia* populations are in panmixia, suggesting that mating system of the species has not been affected by habitat fragmentation. This is supported by the findings from the mating system parameters (Ritland 1989; 2002), which suggest that the majority of progeny from site 20, and to a slightly lesser extent, site 77, are outcrossed, with true selfing and biparental inbreeding accounting for only a small percentage of crosses. The low rate of true and biparental inbreeding estimated by these statistics was similar to that observed in the paternity analysis, and is also similar to selfing levels recorded under orchard conditions (Ito & Hamilton 1969; Sedgely et al. 1990). Of the outcrossed progeny, site 77 possessed a much higher proportion of full sibs than site 20,

suggesting that a small number of trees are dominating reproduction in this site. Both of these sites were of comparable area, however remnant vegetation in site 77 was substantially less contiguous than in site 20, with several mass flowering trees on the patch edges or immediately outside the patch potentially providing the majority of the available pollen pool in this site. Similar results were observed by Fuchs et al. (2003), where spatially isolated individuals of *Pachira quinata*, a tropical dry forest tree of Costa Rica, were observed to produce a greater proportion of singly sired fruits compared with individuals in undisturbed forest.

Mean inter-tree distance between mothers and assigned fathers in site 20 was observed to be shorter than between mothers and all possible fathers, suggesting that a significant number of crosses in this site occurred between nearby trees. In a study into seed set in the tropical forest tree *Shorea siamensis* (Dipterocarpoideae), Ghazoul et al. (1998) observed that *Trigona* bees tended to move between proximal trees more frequently than between distant trees, supporting the results observed here. In site 20, trees that fathered nuts were also more likely to be contributing a larger proportion of the pollen pool at the time of flowering of the mother than the average tree. This was not observed to be the case in site 77.

Inter-site pollen flow as detected by the paternity analysis was relatively high, with 12% of the progeny having fathers allocated from outside the site. This percentage is surprisingly high given the reasonable distance of 2.8 km between the two sites. Mean distance between all *M. integrifolia* study sites and their closest known neighbouring site was 1.2 km, suggesting that pollen transfer between sites may be relatively frequent. While the study sites selected for this study were purposely clumped,

many of the known *M. integrifolia* populations possess a similarly clumped distribution and may be behaving as metapopulations. Genetic drift and inbreeding levels in many *M. integrifolia* sites may potentially be buffered therefore by frequent gene flow between populations.

Gary et al. (1972) found that apiary *Apis melliera* bees predominantly foraged within one kilometre of hives in a macadamia orchard in Hawaii. Similar results were obtained by Steffan-Dewenter and Kuhn (2003) where *A. mellifera* dances were decoded to study the foraging ecology of the species in differently structured landscapes. Overall mean foraging distance was 1526 ± 37 m and did not differ significantly between simple and complex landscapes. Foraging distances of pollen-collecting bees was significantly larger in simple (1743 ± 95.6 m) than in complex landscapes (1543 ± 71 m). These distances are still substantially shorter than those observed in this study however. In other plant species, pollen dispersal distances have been seen to exceed pollinator flight distances (e.g. Ellstrand 1992; Fenster 1991; Rasmussen & Brødsgaard 1992) due to pollen carryover (Levin 1981), which may also be the case for *M. integrifolia* based on the results of this study. Large insect pollinators possess a greater capacity for carryover than smaller pollinators (Ghazoul et al. 1998), making *A. mellifera* bees more likely candidates for the long-distance pollen transfer observed in this study than *Trigona* sp.. A study by Gross and MacKay (1998) into *A. mellifera* foraging behaviour in *Melastoma affine* (Melastomataceae) populations in the wet tropics system of Northern Queensland observed, however, that honeybees never gathered pollen from anthers but rather removed previously-deposited pollen from stigmas. Pollen carryover by honeybees was also not significant

for this species as pollination success (fruit set) was recorded for less than 10% of *A. mellifera* visits to virgin flowers.

Confidence in paternity assignment was relatively low, at only 80-95% for most offspring. Ideally, the majority of offspring should have confidence in paternity exceeding 95%. To achieve this more markers would need to be run, however this was not possible within the time-frame of the project. One other factor that should be taken into consideration when assessing the validity of these results is the intermittent gametic disequilibrium between loci in sites 20 and 77. The categorical allocation technique used here assumes that alleles are independently inherited, and a violation of this assumption may result in skewed likelihood ratios, falsely inflating the likelihood probabilities of putative fathers sharing multiple associated alleles with offspring (Chakraborty & Hedrick 1983). The cumulative exclusion probability for the marker set as determined by the program FaMoz (Gerber et al. 2003) is quite high however at 0.993, and so the gametic disequilibrium observed here is considered to have a minor impact on paternity assignment.

5.4.2 Breeding System

M. integrifolia was not observed to possesses an optimal crossing distance under wild conditions. In contrast, Chrome and Irvine (1986) observed that crosses between populations of the Australian rainforest tree species *Syzygium cormiflorum* (Myrtaceae) produced more fruit than those within populations. Fertilisation success of open-pollinated racemes was at the same level as all controlled crosses. This differs from studies performed on the species under orchard conditions by Ito et al. (1983),

Sedgley et al. (1990) and Wallace et al. (1996), where fruit-set increased following hand-pollination, and possibly suggests that in wild populations, resources, rather than pollen, may be the major factor limiting fruiting. This is further supported by the finding that selfing produced no fewer fruit than outcrossing, despite the partial self-incompatibility system identified in the orchard studies. Further investigation of resource limitation on the species could be undertaken by repeating the experiment on the wild trees under cultivation as part of the National Macadamia Germplasm Conservation Program (Hardner 2004).

5.4.3 Conclusions

Fruit set in wild populations does not appear to be pollen-limited. It has been suggested by several previous studies that the introduced honeybee *A. mellifera* poses a threat to many native Australian pollinators and plant species by altering natural pollination and increasing inbreeding (England et al. 2002; Paton 1993; Vaughton 1996), however this does not appear to be the case for *M. integrifolia*. In contrast, the longer foraging distances and potentially greater capacity for pollen carryover of *A. mellifera* may be important in reducing genetic drift in otherwise isolated populations (Dick 2001). Abundance of *T. carbonaria*, but not *A. mellifera*, in macadamia orchards in Eastern Australia was also observed to be significantly positively correlated with the extent of surrounding eucalypt vegetation (Heard & Exley 1994), potentially heightening the role of *A. mellifera* as an important pollinator in severely fragmented populations where surrounding vegetation is limited.

This is the first study to have investigated the breeding and mating systems of *M. integrifolia* in the wild. Little evidence was observed to suggest that the mating system of *M. integrifolia* populations has been affected by habitat fragmentation. Inbreeding depression is unlikely to be a threatening process in at least medium-sized fragments of *M. integrifolia*, with high outcrossing rates observed in both sites 20 and 77. In addition, the high levels of pollen flow detected between sites 20 and 77 suggest that fragmentation may not necessarily lead to complete isolation of populations, and may buffer small sites from loss of genetic diversity via drift. This study provides strong foundations in assessing the impact of fragmentation on *M. integrifolia* populations, however further research into the mating system of the species incorporating both fragmented and intact populations would greatly improve evaluations of population viability.

Chapter 6 Fragmentation Consequences for Genetic Diversity and Spatial Genetic Structure

6.1 Introduction

Habitat fragmentation, and the associated reductions in population size and increase in isolation, may produce an increased susceptibility to both demographic and genetic hazards (Gilpin & Soule 1986; Young et al. 1996). While demographic instability will contribute, risk of extinction in the long term may be most affected by genetic factors, in particular the loss of genetic diversity via genetic bottlenecks (population size constrictions, Frankham et al. 2002) and drift (Charlesworth & Charlesworth 1987; Ellstrand & Elam 1993). The severity of genetic bottlenecks related to habitat fragmentation is determined by the extent and duration of the reduction in population size (Barrett & Kohn 1991), where populations that suffer a significant decrease in size and remain small for many generations are highly susceptible to loss of allelic diversity and random genetic drift (Barrett & Kohn 1991; Ellstrand & Elam 1993).While the evolutionary significance of reduced genetic diversity remains uncertain (Charlesworth & Charlesworth 1987), on theoretical grounds genetic diversity is considered vital for allowing both short-term adaptation to environmental fluctuations and long-term evolutionary change (Barrett & Kohn 1991; Frankel & Soule 1981; Schaal et al. 1991) and is therefore an important issue in determining population viability.

Due to their sedentary nature and the spatial limitations of seed and pollen dispersal, plant species are also capable of developing strong spatial genetic structure, i.e.

non-random distribution of genotypes in space (Vekemans & Hardy 2004). The strength and spatial magnitude of genetic structuring can influence and is influenced by a variety of factors including selection and historical processes such as gene flow and geographic barriers to gene movement (Epperson & Li 1996; Epperson & Li 1997). Generally, however, spatial structuring of genotypes is predominantly a consequence of limited seed and pollen dispersal (Degen et al. 2001a; Doligez et al. 1998; Epperson & Li 1997; Vekemans & Hardy 2004). Differences in spatial genetic structure associated with fragmentation are therefore likely to be as a result of changes to these processes, which can also have consequences for effective population size and levels of genetic diversity (Wright 1931).

Spatial genetic structuring at the inter-population level is usually the result of historical factors and gene flow/genetic drift (Dutech et al. 2004). The increased isolation and reduction in population size that typically follows habitat fragmentation can result in reduced inter-remnant gene flow and inflated levels of genetic drift, creating high genetic divergence between populations and loss of genetic diversity (Latta & Mitton 1997). It has been suggested that one or two migrants between populations per generation can theoretically counteract inbreeding depression (Newman & Tallmon 2001), however even 5-20 migrants per generation may not prevent loss of genetic diversity within and differentiation between populations (Lacy 1987).

In this chapter, the impact of habitat fragmentation on the level and distribution of genetic diversity within and between sites is investigated. Implications for population viability are also discussed.

6.2 Methods

Estimation of genetic diversity, within-site spatial genetic structure and population differentiation were undertaken in this study. Details of sampling, molecular techniques and data analysis for each of these analyses is outlined below.

6.2.1 Genetic Diversity

Sampling for estimation of genetic diversity included all individuals in the sampled area of each of the ten study sites where possible (1009 samples total for codominant markers, 917 samples for dominant markers). Given the relatively short length of time since Australian settlement by Europeans, many of the adult trees in the study sites are likely to be relicts from prior to habitat fragmentation. Similarly, evidence from aerial photographic records suggests that all small and medium study sites have been fragmented for in excess of 50-60 years. It is therefore extremely likely that a significant proportion of juvenile plants in these sites are the consequence of post-fragmentation mating. Differences between adult and juvenile cohorts have consequently been used in tests of the effect of fragmentation on genetic diversity and fine-scale spatial structure, however it should be kept in mind that other processes may also be involved in generating any differences in genetic parameters between the two cohorts. Based on observations that plants less than two metres in height rarely flower, individuals falling into this category were classified as juveniles, with trees above this height classed as adults.

As described in Section 3.2.1.2.4, all samples were initially run for the dominant RAF primer B11 and later for the codominant microsatellite and RAMiFi loci *STMS1*, *STMS11*, *B11.043mi*, *B11.072mi* and *A06.100mi*. Given the issues reported in Chapter Three regarding locus *B11.043mi*, this marker was excluded from further analyses.

Genetic diversity within sites and cohorts was estimated using Popgene Version 1.32 (Yeh & Boyle 1997). Observed heterozygosity (H_o), and gene diversity (H_e, expected panmictic heterozygosity) were calculated from the microsatellite data, and Nei's (1973) gene diversity (h) and the proportion of polymorphic loci (P) was estimated from the RAF data. Allelic richness per sample (Rs), a standardised estimate of allelic diversity independent of sample size (Leberg 2002), was calculated using the program FSTAT (Goudet 2001). Differences in allelic richness, observed heterozygosity and gene diversity among cohorts and site classes were also tested using the function available in FSTAT. This analysis eliminates bias through the use of permutation tests on samples weighted by size (Goudet 2001), and for the tests performed here 10000 permutations were conducted. Tests were not available in FSTAT to compare Nei's (1973) gene diversity or the proportion of polymorphic loci between groups, and for these a REML linear mixed model analysis was performed with the fixed factors of site class, cohort, and the interaction between the two, with the random factor of site class × site.

Tests were also conducted to determine if study sites had experienced recent reductions in effective population size (Ne). The program Bottleneck (Cornuet & Luikart 1997) was used to detect significant heterozygosity excesses as an indicator of reduction of Ne (Luikart et al. 1998). A two-phased model of mutation was used,

an intermediate model between the Infinite Allele Model and Stepwise Mutation Model that is more appropriate for most microsatellite data (Luikart et al. 1998). Significance was determined using a Wilcoxon sign-rank test, providing relatively high power without the need for a large number of polymorphic loci.

6.2.2 Within-site Structure

The same individuals used in the assessment of dominant marker genetic diversity estimates were also utilised here (346 juveniles and 521 adults across all ten study sites, Table 3.1). Spatial Genetic Software (SGS) Version 1.0c (Degen et al. 2001b) was used to calculate the correlation between spatial distribution and genetic distance of the individuals. The program uses Tanimoto genetic distance (D) (Deichsel & Trampisch 1985), an index equivalent to the Jaccard index (Salgueiro et al. 2004), and allows visualisation of spatial genetic patterns by plotting spatial distance classes against mean genetic distance (D_r). Tanimoto genetic distance was estimated between individuals in each site at spatial distance classes of 40 m increments. This class size was selected as smaller increments frequently resulted in fewer than 30 pairwise comparisons per distance class, which jeopardises statistical robustness (Cavers et al. 2005; Legendre & Fortin 1989). Significance values were verified using permutation tests (n = 1000) based on Monte-Carlo simulation to evaluate deviation from a spatially random distribution of D, against the null hypothesis of no spatial structure. Statistical testing of the spatial autocorrelation results was performed using a REML linear mixed model analysis with the fixed factors of site class, distance class, cohort, and the interactions between these. Site class × site was included as the random factor.

6.2.3 Population Differentiation

Analysis of population differentiation was based on the genetic data used to estimate inbreeding coefficients (Section 3.2.1.2.1; a total of 409 juveniles and 600 adult individuals across all ten study sites). Individuals were genotyped for the two RAMiFi markers *B11.043mi*, *B11.072mi* and *A06.100mi*, and the two microsatellite markers (*STMS1* and *STMS11*) of Vithanage et al. (1997).

Population differentiation (F_{ST}) and gene flow (Nm) between sites was estimated using the software Popgene Version 1.32 (Yeh & Boyle 1997). Given the large distance between the Amamoor and Samford sites (> 200 km), gene flow between the two (within a single generation) is considered highly unlikely, and so measures of F_{ST} and Nm were estimated for sites within each region but not between. To evaluate the impact of fragmentation on population differentiation and gene flow, F_{ST} and Nm were estimated for sites within each site class and cohort. A general ANOVA was performed for each measure to test the effects of site class, cohort, and the interaction between site class and cohort.

Correlation between mean inter-site distance and F_{ST} and Nm was analysed using simple linear regression. Pairwise comparisons of population differentiation and gene flow between the three Samford sites were statistically analysed using G tests with the William's Correction (Sokal & Rohlf 1995).

6.3 Results

6.3.1 Genetic Diversity

Genetic diversity statistics are summarised in Table 6.1. All four microsatellite loci were polymorphic in all 10 sites across the two regions. Allelic richness was significantly ($P = 0.044$) greater in large sites (6.72) than in medium and small sites (6.08 and 5.67, respectively). No difference was observed overall between cohorts. Neither heterozygosity nor gene diversity were significantly related to site class or cohort ($P = 0.765$ and $P = 0.287$, respectively). Similar results were observed for h, with no significant relationship between diversity and site class, cohort, or their interaction, however the percent of polymorphic dominant loci was significantly related with cohort, with adults being more polymorphic on average across the sites (effect = 3.075, s.e.d. = 9.462, Table 6.2).

Table 6.1 Genetic diversity summary statistics based on microsatellite and RAF data, s.e.m. in parenthesis. Rs = allelic richness; A_E = effective number of alleles per locus; H_o = observed heterozygosity; H_e = gene diversity (expected panmictic heterozygosity); h = Nei's (1973) gene diversity; P = percentage of polymorphic RAF loci. Rs, A_E, H_o and H_e were estimated from microsatellite data; h and P were estimated using dominant RAF data.

Site class	*Site*	*Rs*	*A_e*	*H_o*	*H_e*	*h*	*P*
Small	8	5.95 (0.64)	4.20 (0.68)	0.78 (0.04)	0.75 (0.04)	0.18 (0.03)	70.45
	70	5.34 (0.63)	2.60 (0.89)	0.53 (0.14)	0.50 (0.13)	0.18 (0.03)	59.09
	76	5.71 (0.30)	3.54 (0.53)	0.71 (0.05)	0.71 (0.04)	0.22 (0.03)	75.00
Medium	20	5.95 (0.45)	3.52 (0.32)	0.74 (0.03)	0.71 (0.03)	0.21 (0.03)	81.82
	77	5.12 (0.15)	3.13 (0.20)	0.76 (0.03)	0.68 (0.02)	0.22 (0.03)	84.09
	120	6.96 (0.19)	3.29 (0.32)	0.69 (0.02)	0.70 (0.03)	0.16 (0.03)	68.18
	134	6.29 (0.62)	3.38 (0.49)	0.69 (0.07)	0.69 (0.05)	0.15 (0.03)	54.55
Large	128	6.81 (0.35)	4.19 (0.44)	0.76 (0.03)	0.76 (0.03)	0.18 (0.03)	77.27
	129	6.69 (0.61)	3.75 (0.44)	0.70 (0.03)	0.73 (0.03)	0.20 (0.03)	81.82
	131	6.66 (0.37)	3.83 (0.21)	0.74 (0.01)	0.74 (0.02)	0.21 (0.03)	77.27
Overall		7.31 (0.37)	4.47 (0.55)	0.72 (0.19)	0.77 (0.28)	0.23 (0.02)	100.00

Table 6.2 Significance values for Wald tests. h = Nei's (1973) gene diversity; P = percentage of polymorphic RAF loci. Degrees of freedom and Wald/df are shown in brackets. Significance is based on results from models including only the minimum number of terms: * = significant in final model at $P<0.05$; ^{X} = aligned with one or more levels of other factors in the model.

Fixed term	*h*	*P*
Site class	0.714 (2, 0.34)	0.100 (2, 2.31)
Cohort	0.325 (1, 0.97)	0.042 (1, 4.16) *
Isolation	0.051 (1, 3.82) ^{X}	0.129 (1, 2.30)
Site class x cohort	0.355 (2, 1.04)	0.559 (2, 0.58)
Site class x isolation	0.731 (2, 0.31) ^{X}	0.075 (2, 2.59)
Cohort x isolation	0.008 (1, 7.02) ^{X}	0.257 (1, 1.29)
Site class x cohort x isolation	0.235 (2, 1.45) ^{X}	0.612 (2, 0.49)

Tests for heterozygosity excess revealed a significant reduction in effective population size for most sites, however no apparent difference was observed in heterozygosity excess between site classes or cohorts (Table 6.3).

Table 6.3 One tailed probability for heterozygosity excess among sites and cohorts. * = significant at P<0.05.

Site class	*Site*	*Cohort*	
		Juveniles	*Adults*
Small	8	0.031*	0.906
	70	0.438	0.969
	76	0.906	0.063
Medium	20	0.031*	0.031*
	77	0.563	0.031*
	120	0.063	0.969
	134	0.563	0.031*
Large	128	0.031*	0.938
	129	0.031*	0.156
	131	0.063	0.031*

6.3.2 Spatial Genetic Structure

6.3.2.1 Within-site Structure

Site class and distance class were both significantly related to pair-wise genetic distance, however no differences were observed between cohorts or for any of the interactions (Table 6.4). Spatial genetic structure was observed for all site classes, with spatially proximate individuals being more genetically similar on average relative to the site mean (Figure 6.1). Mean genetic distance between individuals across all distance classes was similar between large and medium sites, while individuals in small sites possessed significantly lower genetic distances on average (Figure 6.1). Investigations into the spatial genetic structure of the two sites (20 and 77) used in the paternity analysis in Chapter 4 also confirmed that spatial structuring for adults appears to be present in these sites (Figure 6.2).

Table 6.4 Wald test results for the relationship between Tanimoto genetic distance and the fixed factors of site class, spatial distance class and cohort. * = significant at P<0.01; $^{+}$ = not significant in the final model.

Fixed term	*d.f.*	*Wald/d.f.*	*Chi pr*
Site class	2	21.09	<0.001*
Distance class	5	4.63	<0.001*
Cohort	1	2.34	0.126
Site class x distance class	8	0.78	0.620
Site class x cohort	2	0.39	0.677
Distance class x cohort	5	0.8	0.547
Site class x distance class x cohort	6	2.04	0.050 $^{+}$

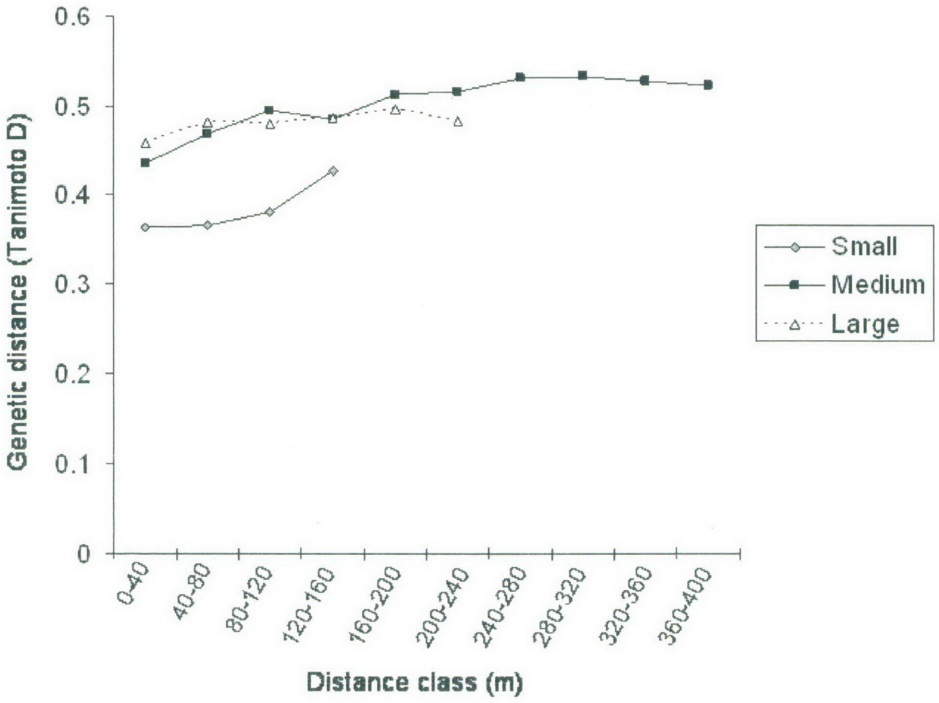

Figure 6.1 Relationship between Tanimoto genetic distance and spatial distance for the three site classes.

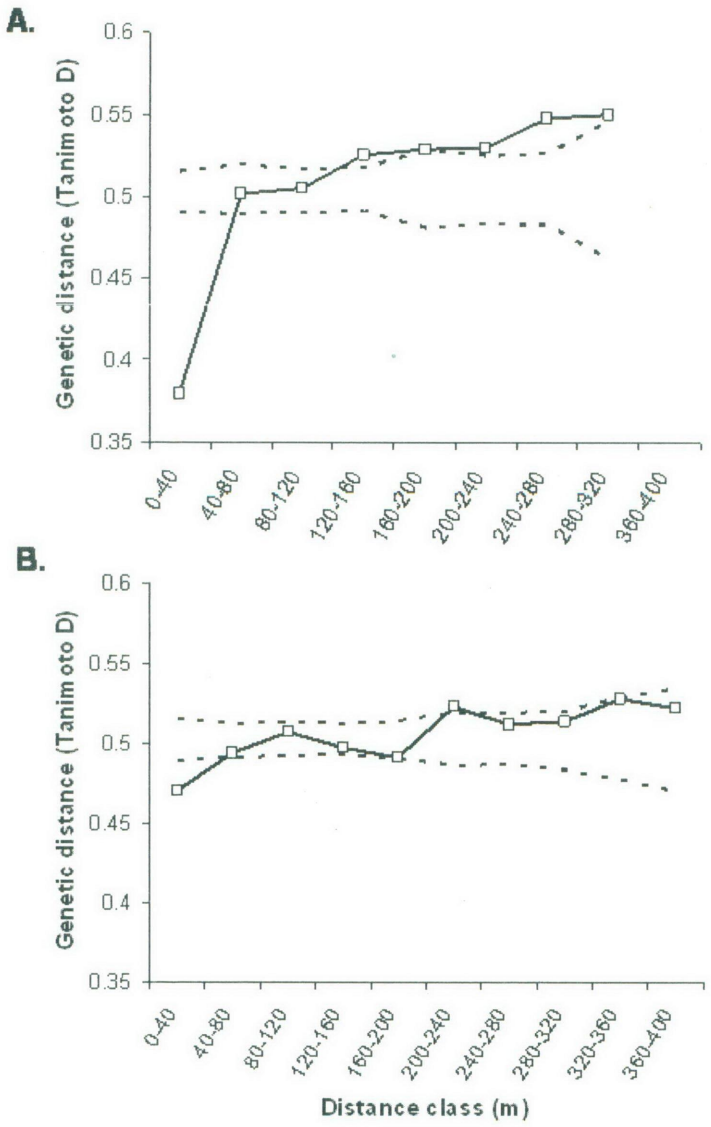

Figure 6.2 Relationship between genetic distance and spatial distance in sites 77 (graph A) and 20 (graph B). Dotted lines represent the upper and lower 95% confidence limits. Significant deviations in genetic distance from the site mean occur when the observed genetic distance (solid line) falls outside of the confidence limits.

6.3.2.2 Population Differentiation

Mean F_{ST} and Nm values for the Amamoor sites grouped by site class are presented in Table 6.5. F_{ST} was observed to be significantly related to site class (Table 6.6), with small sites possessing the greatest amount of differentiation, followed by medium and then large. However, F_{ST} was not found to be significantly related to cohort or the

interaction between site class and cohort. Analysis of Nm revealed similar results, with the mean number of migrants per generation increasing significantly with increasing patch size, however no difference was observed between cohorts (Table 6.7). Genetic differentiation and estimates of gene flow both appear to be unrelated to mean spatial distance between sites ($F_{1,22}$=0.12, P=0.736 and $F_{1,22}$=0.01, P=0.907, respectively).

Table 6.5 Mean sample size, distance between sites, F_{ST} and Nm for small, medium and large sites in the Amamoor region.

Site class	*Mean sample size*	*Mean distance (m)*	*Mean F_{ST}*	*Mean Nm*
Small	178	600	0.132	1.65
Medium	164	500	0.046	5.18
Large	538	625	0.030	8.05

Table 6.6 ANOVA results for Amamoor F_{ST}.

	d.f.	*m.s.*	*Chi pr*
Site class	2	0.0625	<0.001
Cohort	1	0.0043	0.378
Site class x cohort	2	0.0013	0.783

Table 6.7 ANOVA results for Amamoor Nm.

	d.f.	*m.s.*	*Chi pr*
Site class	2	4.6825	<0.001
Cohort	1	0.5282	0.247
Site class x cohort	2	0.4425	0.340

Pairwise comparison of F_{ST} and Nm between the three Samford sites (Table 6.8) revealed slightly higher but not statistically significant differentiation (G=0.0067, P=0.99) and significantly lower mean number of migrants between site 76 and the other two sites.

Table 6.8 Mean sample size, F_{ST} and Nm for the comparison between site 20 and 77, and 20 and 76.

Site comparison	*Mean sample size*	*Mean distance (m)*	*Mean F_{ST}*	*Mean Nm*
20 and 77	368	2500	0.0150	16.3711
20 and 76	320	3000	0.0628	3.7289
76 and 77	262	5500	0.0619	3.7917

6.4 Discussion

6.4.1 Genetic Diversity

Heterozygosity in populations may be used as a measure of the capacity to respond to selection immediately following a genetic bottleneck (Allendorf 1986), and the absence of any differences between site classes or cohorts suggests therefore that short-term fitness of fragmented *M. integrifolia* sites has not been compromised. This is further reinforced by the finding that reductions in effective population size appear unrelated to cohort and site class, suggesting that small and medium sites have not undergone bottleneck events associated with clearing and fragmentation. Reports of positive correlations between heterozygosity and population size are much rarer than those for number of alleles or percentage polymorphic loci (Ellstrand & Elam 1993; Frankham 1996; but see Godt et al. 1996; Luijten et al. 2000). In a similar study into the genetic impact of habitat fragmentation on the Australian rainforest tree *Elaeocarpus grandis* (Elaeocarpaceae), Rossetto et al. (2004) observed contrasting results to those observed here with higher proportional loss of heterozygosity among younger cohorts in fragmented populations (a likely consequence of isolation). Lienert et al. (2002) also found nearly significantly reduced observed

heterozygosity on distant islands of the long-lived perennial *Swertia perennis* (Gentianaceae) regardless of population size, indicating the relatively pronounced effects of habitat fragmentation on the genetic variability of individual plants.

Levels of allelic diversity may determine a population's ability to respond to long-term selection over many generations, and ultimately the survival of the population (Allendorf 1986). The lower levels of allelic diversity observed in the small and medium sites examined in this study may point to small and medium fragments being at greater risk of local extinction in the long term, however, the difference in allelic diversity between fragmented and intact sites, while statistically significant, may not be great enough to be biologically significant. Decreasing allelic diversity with population size has been observed in many other fragmented species including the rainforest tree species *Swietenia humilis* (Meliaceae) (White et al. 1999) and the perennial herb *Gentiana pneumonanthe* (Gentianaceae) (Raijmann et al. 1994). In contrast, numerous studies into genetic diversity of tree species have also observed no difference between fragmented and intact populations (e.g. Dayanandan et al. 1999; Rossetto et al. 2004). Broadhurst and Coates (2004) found that levels of genetic diversity appeared to be unrelated to population size in three Banksia species (Proteaceae), *B. oligantha*, *B. cuneata* and *B. ilicifolia*. Similarly, in a review of neotropical tree species, Lowe et al. (2005) found that the majority of species (nine out of 13) displayed no significant difference in genetic diversity between fragmented/logged populations and control populations. One possible reason for these observations may be the relatively short period of time since fragmentation for many of these species relative to their lifespan (e.g. Dayanandan et al. 1999). For species such as this, trees that were probably present prior to anthropogenic disturbance

are likely to have been sampled, and results therefore may not reflect loss of diversity associated with fragmentation, but pre-existing genetic structure (Aldrich et al. 1998; Broadhurst & Coates 2004; Collevatti et al. 2001). Very few investigations into the impact of fragmentation on genetic diversity levels in tree populations have been observed to account for this issue by analysing potential pre- and post-fragmentation cohorts in addition to site class, as performed in this study. In addition, most previous studies have focussed on population size only, implicitly assuming the same degree of isolation for all populations and possibly overlooking potentially significant genetic consequences of fragmentation. In a study by Lienert et al. (2002), reduced allelic diversity was observed only in both small and isolated populations of *S. perennis*. Similar results were found by Hall et al. (1996), where a negative relationship between genetic variability and geographic isolation was observed for the tree species *Pithecellobium elegans* (Fabaceae). In contrast, isolated populations of *Silene regia* (Caryophyllaceae) were not found to be genetically depauperate, possibly due to long-distance pollen dispersal by hummingbirds (Dolan 1994).

6.4.2 Spatial Genetic Structure

All sites regardless of size class were observed to possess within-site spatial genetic structure, with spatially proximate plants being more genetically similar than on average. Gene flow within these sites is therefore locally restricted (Wright 1943, 1978). In Chapter 4 it was observed that pollen flow within sites 77 and 20 most commonly occurred at distances of 115 m on average, slightly greater than the distances associated with spatial genetic structuring seen here. Genetic isolation by distance within plant populations may be more strongly influenced by seed

dispersal, however, than by pollen flow (Hamilton 1999), and even with random mating, local spatial genetic structure can be generated due to locally restricted seed dispersal (Vekemans & Hardy 2004). Spatially limited seed dispersal can also contribute to clumped distributions of individuals (Hardesty et al. 2005), potentially resulting in limited effective population size, and subsequently reduced genetic diversity (Wright 1931) and significant genetic differentiation among populations (Cardoso et al. 1998; Dutech et al. 2002). The absence of differences in spatial genetic structure between site classes or cohorts of *M. integrifolia* suggests, however, that patterns of within-site gene flow have not been altered significantly by fragmentation.

Along with selection and mutation, gene flow among populations may counteract loss of genetic diversity and its associated negative effects on fitness (Wright 1931). In this study, population differentiation was positively related to patch size and inter-site gene flow was negatively associated with patch size, despite similar spatial distances between sites. While pollinators appear to be active within small sites, as suggested by the high level of nut production observed in these sites in Chapter 3, the above finding may indicate that small sites are less attractive or less visible to long-distance pollinators. However, neither differentiation or gene flow are significantly associated with cohort or the interaction between site class and cohort. Because the adult cohort is assumed to have been present prior to fragmentation, this may suggest that the differentiation observed between the site classes is unrelated to fragmentation, and is possibly associated then with pre-fragmentation spatial genetic structure at the landscape level and/or natural barriers to gene flow, as appears to be the case in the Samford region.

In a review of fragmented and logged neotropical forest tree species, Lowe et al. (2005) report that two of the three studies that examined genetic differentiation within adult populations of fragmented species found decreased differentiation in impacted populations. For example, pollen dispersal in the Amazonian canopy emergent *Dinizia excelsa* (Fabaceae) was found to be much more extensive in remnant forest patches than in undisturbed forest, despite an absence of native pollinators (Dick 2001). The high degree of gene flow was attributed to genetic rescue by Africanized honeybees (*Apis mellifera scutella*) which has longer foraging distances than the native bee pollinators (Dick et al. 2003). Consequently, high levels of genetic diversity were maintained across both fragments and intact forest. In another study by White et al. (1999), direct and indirect measures of gene flow in fragmented and continuous stands of the tropical dry forest tree species *Swietenia humilis* (Meliaceae) revealed an extensive network of gene exchange up to 5 km. Similarly, gene flow between populations of the temperate forest tree *Acer saccharum* (Aceraceae) was observed to increase following fragmentation, presumably because of enhanced wind flux after fragmentation (Fore et al. 1992).

6.4.3 Conclusions

Small *M. integrifolia* sites display evidence of decreased inter-population gene flow, increased genetic differentiation, reduced allelic diversity and higher genetic similarity between individuals compared to medium and large sites. While the decreased gene flow and greater population differentiation did not appear to be as a consequence of habitat fragmentation, the lower allelic diversity in these sites may compromise their long-term viability by limiting their ability to respond to

selection pressures. Further work is required however to evaluate the long-term implications of this. In the shorter-term, within-site gene flow patterns are appearing to be maintained post-fragmentation based on estimates of fine-scale spatial genetic structure, and heterozygosity levels suggest that individuals within these sites may be capable of responding to selective pressure.

In terms of genetic diversity and structure, habitat fragmentation does not appear to be detrimentally affecting wild *M. integrifolia* populations in the short-term. Despite lower allelic diversity in the small and medium sites studied here, the observed relationship between this and fragmentation is weak, suggesting that longer-term population viability may not be threatened by habitat fragmentation either. Due to the relatively short period of time since fragmentation for most tree species studied, it may be the case that the diversity of fragmented populations is still in decline and differences between intact and fragmented sites may not be observed for many of these species for several more generations (Lienert et al. 2002). Whether this may also be the case for *M integrifolia* is unknown.

Chapter 7 Conclusions

Macadamia integrifolia is currently estimated to become extinct in the next 20-50 years due to land clearing and habitat fragmentation (Briggs & Leigh 1988). This classification, however, has been based primarily on qualitative evidence, with little knowledge about the species in the wild or the ability of the species to survive in fragments. Consequently, it was the aim of this study to assess the implications of habitat fragmentation for *M. integrifolia* population viability. More specifically, it was investigated whether populations of *M. integrifolia* are affected by habitat fragmentation in regards to: (i) population demographic processes; (ii) mating system; and (iii) levels of genetic diversity and spatial genetic structure. The outcomes from these questions are discussed below.

7.1 Have demographic processes been altered in fragmented M. integrifolia populations?

The findings of this study suggest that demographic processes in *M. integrifolia* are indeed altered in populations subjected to habitat fragmention, however the implications of this for population viability are not necessarily negative. Small sites possess healthy recruitment patterns, and the proportion of plants flowering and fruiting, and levels of recruitment in small sites all exceed those in the more intact large sites. No difference in mortality between the different site classes was detected. This study therefore suggests that population growth in fragmented populations may actually be greater than that in larger populations. Longer-term census work is required, however, before this could be more confidently assessed. In the short-

term at least, viability based on population growth parameters appears to be positive for fragmented populations.

7.2 Has the mating system of the species been altered by habitat fragmentation?

Analysis of inbreeding levels within *M. integrifolia* study sites revealed no difference in historical mating system between fragmented and intact sites, with all sites displaying evidence of random mating. Unfortunately the relationship between contemporary inbreeding rate and habitat fragmentation was unable to be discerned given the inability to include multiple site classes in the experiment. Contemporary mating system estimates in two medium sites revealed high levels of outcrossing, however, similar to observations of the species under orchard conditions (Ito & Hamilton 1969; Sedgely et al. 1990), suggesting that contemporary mating system in these sites may not have been significantly affected by fragmentation.

Possibly the most significant finding from the assessment of mating system and gene flow in this study is the observation of pollen movement of up to 2.8 km across a cleared matrix in the Samford Valley, suggesting that *M. integrifolia* can maintain a network of gene flow over a wide geographic area. With increased isolation of populations due to habitat fragmentation, this is significant for the maintenance of genetic diversity levels and therefore estimation of long-term viability of fragmented populations.

7.3 Have levels of genetic diversity and spatial genetic structure been altered by habitat fragmentation?

Evidence from this study suggests that while fine-scale spatial genetic structure and population differentiation do not differ between fragmented and intact sites, allelic diversity has been lost from small sites. The most likely causes of this loss are reductions in population size at the time of fragmentation and the continued maintenance of small populations sizes, and may have serious implications for the ability of small populations to adapt to selection pressure. There is the potential, however, for reintroduction of lost alleles into fragments due to the high degree of inter-population gene flow as suggested by the paternity analyses and population differentiation study, so long as those alleles still exist in neighbouring populations, which may at least partially negate the bottleneck effect.

Levels of observed heterozygosity support the findings above that indicate that historical mating system has not been altered with fragmentation. While this suggests that the ability of populations to adapt immediately following environmental change has not been compromised, further investigation of levels of bi-parental inbreeding in populations is required to determine if heterozygosity is likely to erode in the longer-term.

7.4 Summation

While the implications of habitat fragmentation for *M. integrifolia* have been separated into the above three categories for convenience, these factors are in

reality highly inter-related and should be considered together in assessing overall population viability. This thesis has presented little data to suggest that fragmented populations are not viable in the short-term, with strong population growth rates, high outcrossing rates in medium sites, and an ability equal to that of intact populations to adapt immediately following environmental change. Longer-term implications of fragmentation for population viability are less certain, but again little evidence was found that suggested loss of viability in the long-term. The slightly reduced allelic variation observed in fragmented populations of *M. integrifolia* may lower the ability of fragmented populations to respond to selection pressure in the long-term. The biological significance of this small reduction is unknown however, and any negative implications may be at least partially ameliorated by: (a) the maintenance of inter-population gene flow which may help reduce the effect of genetic drift and possibly reintroduce into populations alleles lost during genetic bottlenecks; and (b) purging of deleterious recessive alleles from populations through multiple generations of inbreeding and selection.

While not possessing all of the attributes of an early successional species e.g. efficient long-distance dispersal and long-lived soil seed bank (Shiferawa et al. 2004), the observed increase in flowering and fruiting associated with higher light levels and the ability of seedlings to remain largely dormant until gap formation suggests that the life cycle of the species relies to some degree on disturbance events. This factor, in addition to an outcrossed mating system with potential long-distance pollen dispersal, long life expectation, and resilience of individual trees to damage via coppicing, may at least partially contribute to the observed ability of *M. integrifolia* to cope with fragmentation and disturbance. Habitat fragmentation is also unlikely to be only a

recent phenomenon for the species, and adaptation to cycles of rainforest expansion and contraction over geological history may also account for some of the observed resilience of the species.

A potential threat that wasn't investigated in this study is pollen flow from orchards into wild populations. The distribution of commercial cultivation of macadamia overlaps completely with the natural distribution of the species, and in some cases, orchards are located immediately abutting remnant wild populations. Pollen contamination from orchards may threaten population viability by flooding populations with pollen of very limited diversity, potentially decreasing the number of effective pollen donors and genetic diversity within these sites drastically. Investigation into the impact of orchards on wild populations is therefore important for determining conservation priorities. While two study sites in this investigation were located adjacent to macadamia orchards (sites 70 and 120), without paternity and parentage analyses in these sites, little could be determined of any potential impact from the orchards on the wild populations. Neither orchard has been established for long enough to have had any detectible impact on juvenile genetic diversity levels, and while seedling recruitment was lower in site 120 than the majority of other medium sites, this is more likely due to heavy weed cover in this site than genetic effects from the adjacent orchard.

Based on the results from this study, fragmented populations of *M. integrifolia* maintain conservation value. In the short-term they appear to be self-sustainable, and may provide vital links for inter-population gene flow across fragmented landscapes. Longer-term population viability, in particular ameliorating loss of genetic

diversity, largely depends on levels of inter-population gene flow, and building on the investigations into gene flow performed in this study should therefore be a research priority.

The findings from this study also suggest that while long-term viability of *M. integrifolia* is uncertain, populations are likely to remain viable substantially longer than the 20-50 years suggested by the formal classification. The major immediate threat to the species may be total removal of populations through land clearing, however most remnant populations are already restricted to unusable land in regard to housing or agriculture, and further clearing is unlikely in many cases. The work performed in this study provides a strong foundation for estimating the impact of habitat fragmentation on *M. integrifolia* population viability, and future research expanding on some of the findings in this study, in particular into the impact of fragmentation on mating system, and inter-population gene flow, will greatly benefit our understanding of this topic.