Chapter 3 Molecular Techniques Used and Marker Assessment

3.1 Introduction

Before the development of molecular markers, the study of genetic variation and mating systems was reliant on the use of quantitative genetic techniques to separate genetic and environmental effects on phenotype (Fins et al. 1992; Wang & Szmidt 2001). While useful for plant and animal breeding where pedigree is known and specific traits are of interest, these techniques lacked the ability to unravel the complex genetic relationships associated with population dynamics. The advent of molecular marker tools in the 1980s, however, provided a means of characterising individuals based on heritable and selectively neutral traits (Sunnocks 2000) and resulted in a revolution in population and evolutionary genetics in areas including the study of gene flow, genetic drift, mating systems and population genetic diversity (Avise 1994; Davies et al. 1999; Lewontin 2002; Parker et al. 1998; Wang & Szmidt 2001).

Since its beginnings in protein electrophoresis, molecular techniques have rapidly advanced and the characterisation of genetic variation from all levels of population and evolutionary process is now possible with a high degree of resolution (Sunnocks 2000; Wang & Szmidt 2001). Large quantities of highly informative genetic information can now be accumulated in little time (Glaubitz & Moran 2000) and advances in computational resources, software and statistical techniques have for the first time allowed complex analysis of large amounts of molecular data (Holsinger & Wallace 2004; Jones & Ardren 2003; Manel et al. 2003; Smouse & Sork 2004).

Of the molecular tools available today, microsatellites (or simple sequence repeats – SSRs) are becoming the marker of choice for population, ecological and fragmentation research due to their ability to detect subtle changes in genetic diversity and inbreeding characteristics (Rossetto et al. 2004). The codominant nature and high variability of microsatellites allows them great utility in this area of research, however microsatellite markers are largely genus specific and development is relatively costly (Peace et al. 2004). A cheaper and more rapid technique for molecular data accumulation are dominant marker tools such as Amplified Fragment Length Polymorphism (AFLP) or Randomly Amplified DNA fingerprinting (RAF) (Waldron et al. 2002). While the dominant nature of these markers limits their utility in some areas of population genetic analysis, their non-species-specific nature and rapid information output still makes them a useful tool for projects where microsatellites aren't feasible (Peace et al. 2004).

This chapter outlines the molecular techniques employed in this study and where they were employed. The microsatellite markers utilised were also assessed to ensure their quality and that no assumptions were violated in the statistics performed. These tests and their results are outlined here.

3.2 Methods

3.2.1 Molecular Techniques

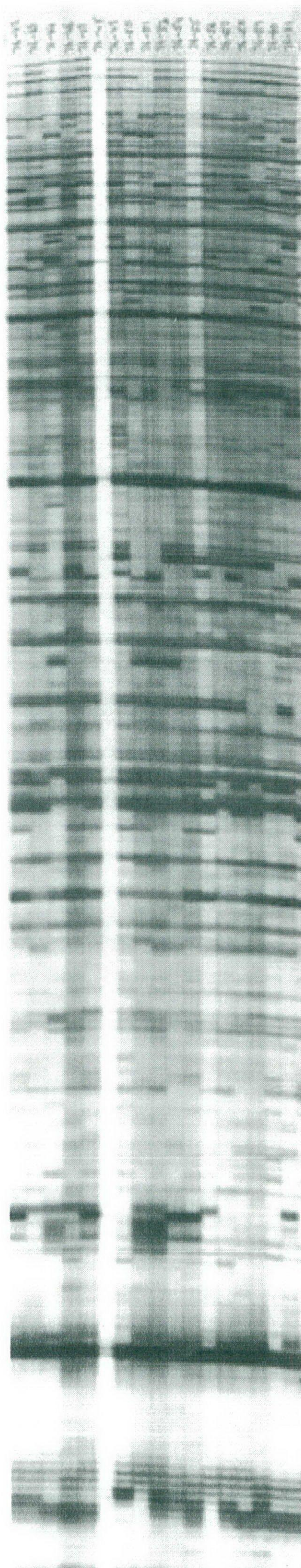
3.2.1.1 Marker selection

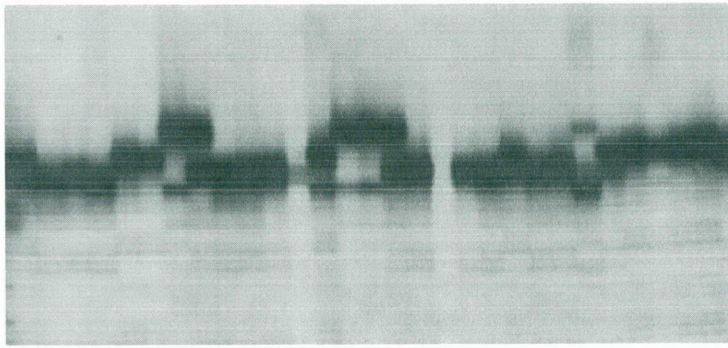
3.2.1.1.1 Dominant markers

At the time that this project commenced, several studies had been performed developing and using molecular markers for *Macadamia* sp. including Randomly Amplified Polymorphic DNA (RAPD) (Vithanage et al. 1997), RAF markers (Waldron et al. 2002) and a limited number of microsatellites (Peace et al. 2004; Vithanage et al. 1997). Despite the greater utility of microsatellites compared to dominant markers such as RAPDs and RAFs, too few microsatellite markers had at that point been developed for *Macadamia* for purposes such as the paternity analyses being undertaken in this study and no scope was available in the project to develop more. RAF markers were successfully being utilised for chromosome mapping, diversity assessment of wild trees, cultivar identification and pollen flow assessment (Peace 2005; Peace et al. 2005; Peace et al. 2003). Consequently, RAFs were identified at the beginning of this study as a promising marker system for broad-scale genetic diversity estimation and paternity assessment in this study due to the convenience they allowed in generating a large amount of information in a short period of time (Peace et al. 2004; Waldron et al. 2002).

RAF (Figure 3.1) is an arbitrarily-primed, PCR-based marker system with electrophoresis on large polyacrylamide gels. This system was desirable as a relatively fast method that produces large numbers of markers per assay, and has advantages over AFLPs

including requiring fewer laboratory steps, tolerating lower quality DNA, and being relatively less expensive per assay (Waldron et al. 2002). Similar to RAPDs, RAF typically uses 10-mer primers and amplifies equivalent DNA fragments usually with dominant inheritance (Waldron et al. 2002). After being run for most individuals across all ten study sites, however, similar findings to those observed with RAPDs, which also rely on random amplification, were observed, and many RAF markers scored were found to be inconsistent or difficult to score reliably. Of the approximately 120 RAF markers originally identified, only 44 were deemed sufficiently consistent for use. This number was too small for most purposes and other options were pursued. The low repeatability of RAF markers observed in this study is in contrast to the results reported by Waldron et al. (2002), who found RAF markers to be highly reproducible for a selection of species. Reasons for this difference in observed reliability are unknown.





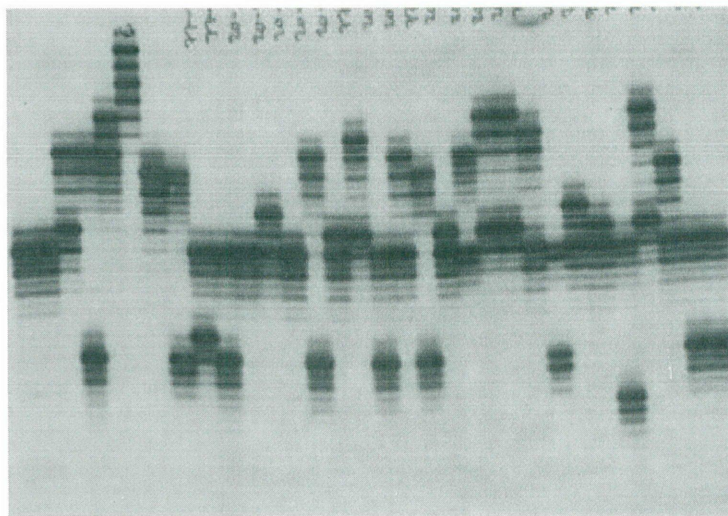


Figure 3.1 (a) Photograph of PCR product from primer B11, showing dominant markers and two RaMiFi loci: *B11.043mi* and *B11.072mi* (Peace et al. 2004); (b) microsatellite locus *MinuS74* (Schmidt et al. 2006); and (c) microsatellite locus *MinuS7* (Schmidt et al. 2006).

3.2.1.1.2 Codominant markers

While RAF markers were initially an attractive choice for broad-scale diversity estimation and paternity assessment, other genetic statistical techniques that require codominant markers (i.e. *F* statistics) were also needed. Consequently, the small number of codominant microsatellite loci available at the beginning of the study were run for a subsample of individuals in each study site. These comprised of two microsatellite markers developed by Vithanage et al. (1997) and three RAMiFi (randomly amplified microsatellite fingerprinting) (Figure 3.1) markers (Peace et al. 2004).

While the majority of markers amplified using the RAF protocol demonstrated dominant inheritance, a number of microsatellite loci were also amplified and have been named RAMiFi markers (Peace et al. 2004). Null alleles were identified early on as a potential issue for these loci and a locus-specific primer was designed for one of these by Peace et al. (2004) to help address this problem. Because RAF markers were being employed as well as RAMiFi in this study, however, it was considered more efficient in terms of time and money to amplify both simultaneously using the same protocol as for the RAF markers (Waldron et al. 2002) and deal with null alleles at the analysis stage.

In addition to codominant RAMiFi markers, eight microsatellites developed by Vithanage et al. (1999) were amplified and screened on polyacrylamide gels using the RAF protocol, for use in estimating F statistics. Of those screened, only two (*STMS1* and *STMS11*) amplified successfully and displayed significant variation.

In 2006 Schmidt et al. generated 33 more microsatellite markers for macadamia (Figure 3.1). Because of the limited utility of the RAF markers for paternity analysis

for the reasons stated in Section 3.2.1.1.1, five of the most variable microsatellites developed by Schmidt were run in addition to the three RAMiFi and two microsatellites mentioned above for all individuals involved in the paternity analysis.

3.2.1.2 Marker application

A number of genetic analyses were undertaken in this study. Given unavailability of a useful number of microsatellite markers at the beginning of the study and issues with RAF reliability, marker application was not straight-forward. Table 3.1 provides a summary of marker application, and is discussed in more detail below.

Table 3.1 Summary of marker application in this study.

Analysis	*Chapter*	*Codominant markers*										*Dominant markers*
		043mi	*072mi*	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*	*MinuS74*	*RAF*
Inbreeding coefficients	5	run but not included in analysis	✓	✓	✓	✓						
Paternity analysis	5	run but not included in analysis	✓	✓	✓	✓	✓	✓	✓	✓	✓	
Breeding system	5											✓
Genetic diversity	6	run but not included in analysis	✓	✓	✓	✓						✓
Spatial genetic structure	6											✓
Population differentiation	6	run but not included in analysis	✓	✓	✓	✓						

3.2.1.2.1 Inbreeding Coefficients

The calculation of inbreeding coefficients requires codominant marker data and the microsatellite and RAMiFi markers available at the beginning of the study were employed for this purpose. All adults and a sample of juvenile plants in each of the ten

study sites were genotyped for the two microsatellite loci (*STMS1* and *STMS11*) developed by Vithanage et al. (1997) and the three RAMiFi loci (*B11.043mi*, *B11.072mi* and *A06.100mi*) described by Peace et al. (2004).

3.2.1.2.2 Paternity Analysis and Mating System Parameters

Paternity analysis in this study was initially planned to be performed using dominant RAF data. However, the limited reliability of these markers as discovered later and subsequent low number of final markers available would have been extremely problematic for the accuracy of paternity estimation. With the generation of additional microsatellite markers by Schmidt et al. in 2006, enough microsatellites were available for use in paternity analysis in this study and were employed accordingly.

Paternity analyses were conducted in 2003 in two adjacent Samford study sites, 20 and 77. Roughly ten to 15 nuts collected from each of seven trees in site 20 and four trees in site 77 were genotyped, along with all adult individuals in both sites observed to flower in the previous season. Individuals were typed for the seven microsatellite loci (Schmidt et al. 2006; Vithanage et al. 1997) and the three RaMiFi loci mentioned above (Section 3.2.1.1.2). Microsatellite data from the progeny arrays was also utilised to estimate Ritland's (2002) mating system parameters for sites 20 and 77.

3.2.1.2.3 Breeding System Analysis

Analysis of *M. integrifolia* breeding system involved performing controlled cross-pollinations between individuals of varying spatial and genetic distance. Genetic distances between all individuals in sites 8, 20, 77 and 134 were initially estimated, and

those suitable for crossing (i.e. were accessible and producing sufficient numbers of racemes in the years of crossing) within the various genetic and spatial distances identified. RAFs were selected as the most appropriate markers for estimating genetic distance as a greater proportion of trees in the study sites of interest had been genotyped at that time using these markers than the available codominant markers. Reliability of the RAF data estimates were later verified using genetic distances estimated from codominant data (*STMS1*, *STMS11*, *B11.043mi*, *B11.072mi* and *A06.100mi*).

3.2.1.2.4 Genetic Diversity

Where possible, all individuals in the sampled area of each of the ten sites were genotyped for the RAF primer B11. This was limited by the discovery of additional individuals after the genetic analyses had been undertaken, as well as the inability to produce PCR product from some individuals. While re-extraction of DNA proved successful for the majority of cases, any samples that failed the second time were excluded from the analysis. Very small seedlings were also omitted from this investigation if removal of leaf material for DNA extraction would have adversely affected future growth and survival.

Following discovery of the limited reliability of the RAF markers, the codominant markers used to estimate inbreeding coefficients (*STMS1*, *STMS11*, *B11.043mi*, *B11.072mi* and *A06.100mi*) were also employed to calculate genetic diversity parameters. Results from both dominant and codominant marker data are presented.

3.2.1.2.5 Within-Site Spatial Genetic Structure

Despite the availability of the microsatellite loci, RAF markers were still necessary for within-site estimation of spatial genetic structure, as sampling of individuals with the RaMiFi and microsatellite markers was not extensive enough to consistently satisfy the statistical criteria of a minimum of 30 comparisons within each spatial class (Degen et al. 2001b). All individuals genotyped for Section 3.2.1.2.4 were included in the analysis.

3.2.1.2.6 Population Differentiation

Estimation of population differentiation was estimated using the codominant markers available at the beginning of the study. Individuals were genotyped for the two RAMiFi (*B11.043mi*, *B11.072mi* and *A06.100mi*) and two microsatellite markers (*STMS1* and *STMS11*) described in Section 3.2.1.2.1 for the calculation of inbreeding coefficients.

3.2.2 Laboratory protocols

Laboratory procedures for DNA extraction, amplification, and marker visualisation were all undertaken as described in the protocols of previous studies. DNA extraction from both leaf and kernel tissue was performed using chloroform extraction/isopropanol precipitation based on a method developed for grape (Thomas et al. 1993) and customised for macadamia (Vithanage et al. 2002). RAF and RAMiFi markers were amplified and run simultaneously using the technique of Waldron et al. (2002), and amplification and visualisation of the microsatellite markers *STMS1* and *STMS11* also followed this protocol. The five microsatellite markers developed by Schmidt et al. (2006) were amplified and run using the protocol described in that work.

3.2.3 Assessment of Microsatellite Markers

Microsatellite markers are considered to be among the most useful and popular molecular markers for population genetic analyses (Lefort 1999; Wallace et al. 1996), however they can suffer from several problems that may negatively affect their utility by violating statistical assumptions (Selkoe & Toonen 2006). Prior to being used to calculate inbreeding coefficients or paternity, the microsatellite markers used in this chapter were therefore tested for genotyping error, null alleles, deviation from panmixia, selective neutrality and gametic disequilibrium. Allele scoring error was calculated as the proportion of allele mismatches between duplicate PCRs of 10-20% of the individuals run for each locus (following Bonin et al. 2004). Null allele frequencies were estimated per locus and study site using the program FreeNA (Chapuis & Estoup in revision). FreeNA estimates null frequencies for each locus and population using the Expectation Maximisation algorithm of Dempster et al. (1977), which has been shown to perform better than the estimators of Chakraborty et al. (1992) and Brookfield (1996) (Chapuis & Estoup in revision). The software also adjusts genotype frequencies in the raw data to account for the calculated null allele frequencies. This adjusted dataset was subsequently used to perform Hardy-Weinberg Exact Tests to assess for panmixia within populations (Guo & Thompson 1992; Haldane 1954; Weir 1990) using the program Genepop on the Web (Raymond & Rousset 1995). Selective neutrality of loci was assessed using the software Detsel (Vitalis et al. 2003) following the method described by Vitalis et al. (2001). Gametic disequilibrium between loci within sites was also tested using the non-adjusted dataset in Genepop. High levels of association between alleles in site 77 led to the hypothesis that spatial genetic structure was present at the site between two gully systems (denoted 77a and 77b). This was further investigated by calculating mean

relatedness (R) values and F_{IS} within each gully using the software Popgene Version 1.32 (Yeh & Boyle 1997) and Genepop on the Web (Raymond & Rousset 1995) respectively.

3.3 Results

3.3.1 Assessment of Microsatellite Markers

Results from tests of the microsatellite loci used in this study are presented below.

3.3.1.1 Genotyping Error

Mistyping rates were estimated for all loci. Error rates ranged from 0 to 4.69% for the seven microsatellites and from 7.98 to 19.57% for the RaMiFi markers amplified (Table 3.1).

Table 3.1 Number of repeated PCRs and percentage error rate for the ten loci run in this study. Loci *MinuS1a*, *MinuS5*, *MinuS7*, *MinuS14* and *MinuS74* were run for individuals in sites 20 and 77 only. The remaining loci were run for all study sites.

	Loci									
	043mi	*072mi*	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*	*MinuS74*
Total no. of individuals typed	1179	1128	1282	867	882	264	279	303	286	271
Total no. of repeated pairs	332	262	170	79	27	25	36	39	37	32
% error rate	19.57	8.65	7.98	1.97	3.70	0.00	2.78	3.85	2.70	4.69

3.3.1.2 Null Alleles and Population Panmixia

Null allele frequencies were extremely high across most sites for locus *043mi* and high in several sites for *072mi*, *100mi*, *STMS1* and *STMS11* (Table 3.2). Results from the Hardy-Weinberg Exact Tests, performed using null-adjusted datasets, are shown in Table 3.3, where most loci were observed to be in panmixia within sites.

Table 3.2 Estimated null allele frequencies across the ten study sites.

Site class	Site	Loci									
		043mi	*072mi*	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*	*MinuS74*
Small	8	0.82	0.06	0.34	0.06	0.34	-	-	-	-	-
	70	0.54	0.00	0.73	0.18	0.00	-	-	-	-	-
	76	0.83	0.00	0.19	0.00	0.20	-	-	-	-	-
Medium	20	0.46	0.38	0.36	0.22	0.05	0.05	0.00	0.00	0.08	0.20
	77	0.64	0.11	0.14	0.18	0.02	0.03	0.00	0.01	0.13	0.09
	120	0.81	0.04	0.11	0.28	0.22	-	-	-	-	-
	134	0.55	0.00	0.39	0.07	0.11	-	-	-	-	-
Large	128	0.61	0.33	0.19	0.22	0.17	-	-	-	-	-
	129	0.61	0.36	0.47	0.41	0.10	-	-	-	-	-
	131	0.58	0.14	0.44	0.21	0.21	-	-	-	-	-

Table 3.3 Site and locus P-values from Exact tests for Hardy-Weinberg Equilibrium. Significant results indicate departure from panmixia. * = significant at P<0.05; ** = significant at P<0.01.

Site class	Site	Loci									
		043mi	*072mi*	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*	*MinuS74*
Small	8	0.141	0.192	0.089	0.175	0.040*	-	-	-	-	-
	70	0.082	0.783	0.886	0.594	1.000	-	-	-	-	-
	76	1.000	0.010**	0.167	0.843	0.249	-	-	-	-	-
Medium	20	0.135	0.017*	0.788	0.298	0.918	0.316	1.000	0.135	0.475	0.004**
	77	0.063	0.929	0.057	0.137	0.741	0.122	1.000	0.001**	0.344	0.740
	120	0.133	0.553	0.263	0.093	0.104	-	-	-	-	-
	134	0.550	0.406	0.690	0.023*	0.509	-	-	-	-	-
Large	128	0.245	0.655	0.354	0.935	0.069	-	-	-	-	-
	129	0.011*	0.074	0.076	0.345	0.023*	-	-	-	-	-
	131	0.033	0.857	0.660	0.121	0.005**	-	-	-	-	-

3.3.1.3 Gametic Disequilibrium

Tests of gametic disequilibrium between loci within each site revealed little evidence for associations between alleles (see Appendix). Significant gametic disequilibrium between loci is the most frequent in site 77. At closer inspection, site 77 appears to possess spatial genetic structuring, with two genetically distinct gully systems. The smallest gully (77a) largely consists of tightly clumped individuals with a high proportion of associated loci (Table 3.4). Mean R values indicate a significantly higher level of relatedness between individuals in 77a compared with both the large gully (77b) and site 20 ($F_{2,6527}$=20.22, P<0.001). Mating system also appears to differ between the two gullies, with F_{IS} values of 0.043 for site 20, -0.286 for 77a and 0.053 for 77b.

Table 3.4 P-values from tests for gametic disequilibrium between loci for gullies 77a and 77b in site 77. * = significant at P<0.05, ** = significant at P<0.01.

77a

	072mi	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*
100mi	0.126							
STMS1	0.001**	0.002**						
STMS11	0.116	0.396	0.304					
MinuS1a	0.000**	0.007**	0.005**	0.126				
MinuS5	0.509	0.143	0.337	1.000	0.131			
MinuS7	0.002**	0.000**	0.004**	0.265	0.000**	0.027*		
MinuS14	0.029**	0.000**	0.015*	0.873	0.002**	0.002**	0.001**	
MinuS74	0.159	0.548	0.633	0.602	0.010**	0.228	0.006**	0.184

77b

	072mi	*100mi*	*STMS1*	*STMS11*	*MinuS1a*	*MinuS5*	*MinuS7*	*MinuS14*
100mi	0.054							
STMS1	0.526	0.008**						
STMS11	0.112	0.165	0.669					
MinuS1a	0.003**	0.026*	0.196	0.809				
MinuS5	1.000	0.964	0.623	0.043*	0.318			
MinuS7	0.014**	0.000**	0.046*	0.715	0.000**	0.080		
MinuS14	0.466	0.600	0.419	0.014**	0.073	0.441	0.106	
MinuS74	0.050*	0.175	0.116	0.507	0.008**	0.281	0.361	0.234

3.4 Discussion

Despite the relatively low technical demands of RaMiFi (Peace et al. 2004), these markers proved problematic for population genetic analyses in this study due to their high mistyping rate and null allele frequency. Allele scoring error was extremely elevated in the RaMiFi markers, potentially due to allelic drop-outs and difficulties identifying RaMiFi alleles from background RAF markers. The mononucleotide repeat motif of *A06.100mi* also complicated scoring, frequently resulting in slight vertical shifts in allele locations between PCRs. The above issues of allelic drop-out, null alleles and background RAF markers interfering with scoring are likely to be largely resolvable for RaMiFi by designing locus-specific primers, however the results suggest that RaMiFi loci with stuttering patterns suggestive of mononucleotide repeats be avoided in future studies as they proved difficult to score.

High genotyping error rate and null allele frequencies are most problematic for studies that require individual identification such as paternity analysis and population size estimation (Paetkau 2003). Removing *B11.043mi* from the paternity analysis therefore resulted in much higher confidence levels than when it was included, despite the associated loss of genetic data. For analyses at the population level such as F_{IS} estimation, these issues are less likely to result in major bias or lost precision (Bonin et al. 2004), however the error rate in *B11.043mi* was deemed so extreme that it was also excluded from these analyses.

Chapter 4 Impact of Fragmentation on Population Demography

4.1 Introduction

Habitat fragmentation is widely recognised as one of the major factors influencing the persistence of species and species assemblages (Henle et al. 1996; Laurance & Bierregaard 1997), and one of the major determinants of the viability of fragmented populations is the maintenance of regeneration patterns (Fuchs et al. 2003). The population dynamic processes of recruitment, mortality, immigration and emigration may be impacted by fragmentation (Jules & Rathcke 1999; Laurance & Bierregaard 1997; Laurance et al. 1998a; Nason & Hamrick 1997; Rathcke & Jules 1993; Turner et al. 1996), the consequences of which can include a shift in the demographic structure of a population (Harper 1977), the number of individuals (Cordeiro & Howe 2003), and plant density and spatial structure (Bleher et al. 2002). Changes in the demographic structure of populations can also have repercussions for pollinator visitation rates and foraging behaviour, and subsequently reproductive performance (Bosch & Waser 1999; Kunin 1993, 1997; Nason & Hamrick 1997; Young et al. 1993). Ultimately, changes to demographic patterns can increase susceptibility of fragmented populations to local extinction from stochastic events or population decline (Lienert & Fischer 2003), and are therefore closely associated with population viability.

This chapter aims to identify the impact of habitat fragmentation on the demography of *M. integrifolia* populations. Section 4.3.1 examines the relationships between the potentially fragmentation-related site attributes of area, isolation, disturbance and

projected foliage cover (PFC), as a framework by which the remainder of the chapter is assessed. Section 4.3.2 explores the impact of fragmentation on population demographic state (an indicator of historic population dynamic patterns) by examining the influence of the site attributes on plant size and density. Finally, the impact of fragmentation on contemporary population dynamics at site and individual levels is investigated in Section 4.3.3.

4.2 Methods

4.2.1 Assessment

4.2.1.1 Site-Level Variates

The site-level variates of isolation and disturbance were assessed for each study site. In this study, isolation was defined as the distance between each study site and the closest forest patch known to contain *M. integrifolia.* Distance was estimated using aerial photography to identify site locations, and topographic maps to determine scale.

Disturbance was estimated based on the index of Ross et al. (2002):

$$D_k = 1\ (E_k \times S_k)$$

where k = the type of disturbance, E_k = the extent (percentage of fragment area) affected by disturbance k, and S_k = the severity (score of intensity on a scale of one to five) of disturbance k. Five types of disturbance identified as relevant to the study sites were individually assessed for each site using the formula above. These were: (i) soil

compaction, (ii) trampling of vegetation by livestock, (iii) herbivory by livestock, (iv) weeds, and (v) the addition of foreign materials (e.g. rocks or dumped materials) (Gross 1995).

4.2.1.2 Plant-Level Variates

To characterise individual plant demographic parameters, assessments were made of: (i) plant size, (ii) plant density, (iii) presence or absence of flowering, (iv) number of racemes per tree, (v) presence or absence of fruiting, (vi) number of fruit per tree, (viii) recruitment, (ix) mortality, and (x) individual projected foliage cover (PFC).

Plant size was assessed by measuring plant height, trunk cross sectional area (CSA), and projected canopy area (PCA). The height of plants less than 50 cm was determined to an accuracy of one centimetre using a standard ruler. All other plants were measured using a Digital Hypsometer Forestor Vertex to an accuracy of 0.1 m. CSA (following Pisanu 2001) was used as an indicator of trunk size in place of the more commonly used diameter at breast height (DBH), as *M. integrifolia* frequently possesses multiple trunks or coppices that cannot be accurately assessed as a single measure by DBH. In 2003, plants over two metres in height at all sites were measured for CSA. Plants less than two metres tall typically possessed thin stems less than one centimetre in diameter that were difficult to measure accurately and were therefore omitted from measurement. DBH of all stems at 1.3m above ground level was determined using a diameter tape. The CSA of each stem was then calculated as the area of a circle with the given diameter, and the cumulative area of all stems per tree determined to give the final CSA figure per tree. PCA was also estimated for all plants over two metres in height. North-South and East-West directional canopy diameters were measured and used to determine the area of an

oval with those dimensions. Significant canopy extensions in directions outside North, South, East or West parameters were also measured and included in the estimate.

Density was estimated using mean nearest neighbour distances (NND) rather than the number of individuals per unit area, as the latter is conceptually clear only when applied to spatially uniform populations (Kunin 1997). For *M. integrifolia*, NND was the most suitable density estimate, as individuals of the species were frequently observed to possess a clumped distribution. The locations of all known plants in each of the study plots were determined in Australian Map Grid Coordinates (as described in Chapter 2). The computer program Biotas (version 1.03.1 alpha) was used to plot sites and calculate mean NND for each site. Within each site, plants were grouped into height classes of 0-0.5 m, 0.5-1 m, 1-5 m, 5-10 m, and 10-50m, and NND calculated among plants within each height class to identify stage-related differences. Similarly, nearest flowering neighbour distance (flowering NND) was calculated for flowering plants in each year. For sites 8, 128, 129 and 131 where plots were used, individuals that were closer to the plot edge than their nearest neighbour were excluded from the analysis as they could potentially have nearest neighbours outside of the mapped area.

Flower monitoring was undertaken at Samford sites 20, 76 and 77 in September 2001, and at all sites in 2002 and 2003. Plants less than two metres in height were very rarely observed to flower with only two instances observed across all sites and years of the study, these cases being previously larger plants that had been reduced in size due to herbivory or other damage. Monitoring of the presence/absence of flowers and the number of racemes per plant was therefore performed for trees taller than two metres only. In January 2002, the presence/absence of fruit and number of fruit per tree were

recorded for all plants greater than two metres in height at the three Samford sites. In 2003 and 2004, this census was undertaken at all ten study sites. Recruitment in the study sites was assessed in May of 2002, 2003 and 2004 for the Samford sites, and 2003 and 2004 only for the Amamoor sites. Mortality was also recorded at this time.

Given the high degree of variation in PFC within sites, this variate was assessed individually for *M. integrifolia* plants over two metres in height as a means of estimating the relative amount of light each plant received. *M. integrifolia* trees in the top canopy stratum were automatically reported as having 0% PFC. For the remaining plants, digital photos were taken of the forest canopy from two metres outside of the drip line of the tree to the North, South, East and West orientation. Using Adobe Photoshop Version 6.0, the photos were manipulated so that all canopy foliage was shaded black, and all sky white (Figure 4.1). The Histogram function was then used to calculate the percentage of black pixels in each photo, which directly translates to PFC. The results from each of the four photos per tree were subsequently averaged to provide a single estimate of PFC per tree. One potential source of error with this technique is that all photos were taken from eye height (approximately 1.6 m above ground level), and PFC therefore incorporates all foliage from this height and above, resulting in a potential overestimation of PFC for trees higher in the canopy strata. This is not believed to be a major issue, however, as the middle stratum was limited in many sites, and photos were taken to avoid this where possible. Foliage cover was not recorded for any plants where canopy structure brought into question the reliability of the measure.

Figure 4.1 Example of manipulation performed to canopy photographs prior to calculating projected foliage cover. (a) original photograph; (b) manipulated photograph, with white sky and black canopy.

4.2.2 Analysis

4.2.2.1 Site-Level Variates

Simple Pearson correlations were calculated to examine the relationship between site isolation and the five different measures of disturbance to reduce the number of correlated variates. The significance of the correlation coefficients were tested following Sokal and Rohlf (1995).

To examine the effect of site class on site attributes, ANOVA were undertaken on the reduced set of site isolation and disturbance classes with a single factor model.

The relationship between site class and PFC, and site class and $\log_e$ flowering NND were both examined with mixed model residual maximum likelihood (REML) analyses that included the fixed factor site class and the reduced set of site attributes, and the random effect of site within site class. To more accurately determine the significance of the main effects, the terms were fitted in several different orders (as per Payne et al. 2005).

Significance was also assessed using the second half of the Wald table of effects, where the effect of individually dropping terms from the full model is shown. This process was followed for all other REML and Generalized Linear Mixed Models (GLMM) tests in this study. Simple Pearson correlation was used to examine the relationship between $\log_e$ flowering NND and PFC.

4.2.2.2 Population Demographic State

Simple Pearson correlations were calculated to determine the relationship between the plant size attributes of height, CSA and PCA to reduce the number of correlated variates. Significance of correlation coefficients was tested as described above.

Analyses were undertaken to examine the relationship between plant height and site class and other site attributes. Individual plant height was logarithmically transformed to normalise the residual variance. A mixed model REML analysis was undertaken with a model that included the fixed factors of site class, compaction, foreign materials and weeds. Site class × site was used as the random factor.

To examine the relationship between plant density and site attributes a mixed model REML analysis was performed. NND was logarithmically transformed to normalise residual variance. The fixed factors included were site class, compaction, foreign materials, weeds and PFC, and site class × site was used as the random factor.

4.2.2.3 Population Dynamics

Due to the distribution of the data, GLMMs were selected as the most appropriate means of testing the impact of site and tree attributes on flowering. A Binomial distribution with Logit link function was used, and the term site class × site was selected as the random factor. Tests for significance between effects were performed on the scale of analysis, and means presented on the scale of measurement. Flowering data from 2001 was not included in the analysis due to the limited number of sites at which monitoring was undertaken in that year.

A GLMM was also used to assess the relationships between site attributes and number of racemes per flowering tree. With a data set including only plants that flowered in each of the two years studied, a GLMM with Poisson distribution, a Logarithmic link function and the term site class × site as the random factor was performed.

To examine the relationship between site attributes and fruiting, a GLMM similar to that for flowering was used, including the term Log_e Flowering NND and with a reduced data set including only plants flowering in 2002 and 2003. The relationships between site attributes and the number of nuts per fruiting tree were examined using the same test as for number of racemes with the addition of log_e flowering NND, and with a data set including only plants observed to produce fruit in 2003 and 2004. Similarly, the relationship between number of nuts per raceme and site and tree attributes were analysed using a GLMM with the same distribution and link function as above, and including a data set comprised of only those plants that produced fruit in 2002 and 2003.

Recruitment in 2003 and 2004 was examined using a REML analysis with the explanatory variate of Log_e number of recruits per hectare, and response variates of compaction; foreign materials; weeds; Log_e total nuts produced per hectare; site class; year; and the interactions between nuts per hectare and year, and site class and year.

Due to the low death rate observed in the study sites, statistical analysis of mortality was not performed; summary statistics are provided instead.

4.3 Results

4.3.1 Site-Level Variates

Significant positive correlations were found among the site attributes of isolation, compaction, herbivory and trampling (Table 4.1). Of these, compaction was the most highly correlated with the other three, hence isolation, herbivory and trampling were excluded from further analyses. No significant differences were found between site classes for any of the three disturbance traits examined (compaction, herbivory and trampling), and mean distance between flowering neighbours (log_e flowering NND) was observed to be unrelated to site class, compaction, foreign materials, weeds and year (Table 4.2).

Table 4.1 Correlation matrix of site attributes. * = significant at P<0.05; ** = significant at P<0.01

	Compaction	*Foreign materials*	*Herbivory*	*Trampling*	*Weeds*
Isolation	0.80**	-0.12	0.66*	0.80**	0.44
Compaction		-0.13	0.90**	0.98**	0.06
Foreign materials			-0.12	-0.04	0.29
Herbivory				0.79**	-0.18
Trampling					0.16

Table 4.2 Significance values for ANOVA: F probability (d.f., variance ratio).

Fixed term	*Compaction*	*Foreign materials*	*Weeds*	*Year*	*Site class*
Site class	0.25 (2, 1.68)	0.16 (2, 2.45)	0.61 (2, 0.53)		
Flowering NND	0.685 (1, 0.17)	0.193 (1, 1.7)	0.077 (1, 3.13)	0.053 (1, 3.74)	0.657 (2, 0.42)

4.3.2 Population Demographic State

4.3.2.1 Effect of Site Attributes on Plant Size

The three measurements of plant size (height, CSA and PCA) were all found to be significantly correlated (Table 4.3). Height was selected as the most appropriate variate to describe plant size as unlike CSA and PCA, height measurements were taken for all plants and in all three years of the study.

All three site classes displayed an inverse J-shaped height class distribution (Figure 4.2). Plant height differed significantly among site classes (Table 4.4), however, with average height increasing with site area. Mean plant size was 3.26 m in large sites, 0.49 m in medium sites and 1.87 m in small sites. Height was significantly and positively related to

compaction and foreign materials [effect = 0.005 (s.e.d.: 0.001) and 0.009 (s.e.d.: 0.0029), respectively].

Table 4.3 Correlation matrix of log height, log CSA and log PCA. All three values are significant at the P<0.01 level.

	Log Height	*Log CSA*
Log CSA	0.86	-
Log PCA	0.81	0.88

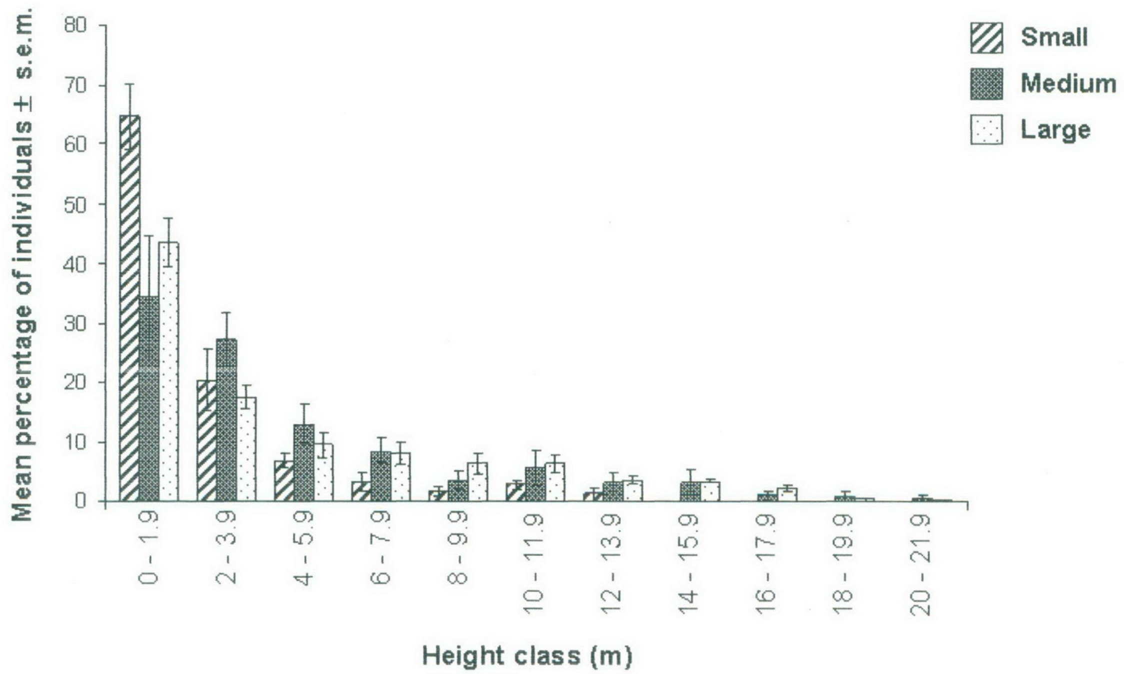


Figure 4.2 Height class distribution (± s.e.m.) for small, medium and large sites in 2004.

Table 4.4 Significance values for Wald tests. Degrees of freedom and Wald/df are shown in brackets. NND = Nearest neighbour distance; PFC = projected foliage cover. 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; ^{XX} = significant only with all other terms in the model; [light shading] = significant positive relationship; [dark shading] = significant negative relationship.

Fixed term	Demographic State		Population Dynamics					
	Height	*NND*	*Flowering*	*No. of racemes per tree*	*Fruiting*	*No. of nuts per tree*	*No. of nuts per raceme*	*Recruitment*
Site class	<0.001 (2, 28.95) *	<0.001 (2, 25.69) *	0.503 (2, 0.69) *	0.146 (2, 1.92) ^{X}	<0.001 (2, 7.13) *	0.062 (2, 2.77) ^{X}	0.977 (2, 0.02)	<0.001 (2, 10.25) *
Compaction	<0.001 (1, 23.11) *	0.248 (1, 1.33) ^{X}	0.71 (1, 0.14)	0.108 (1, 2.58) ^{X}	0.026 (1, 4.95) ^{X}	0.335 (1, 0.93)	0.257 (1, 1.29)	0.505 (1, 0.44)
Foreign materials	0.012 (1, 6.35) *	<0.001 (1, 28.38) *	0.202 (1, 1.63) ^{XX}	<0.001 (1, 14.05) *	0.018 (1, 5.58) ^{X}	0.005 (1, 7.71) ^{X}	0.41 (1, 0.68)	<0.001 (1, 65.25) ^{X}
Weeds	0.002 (1, 9.67) ^{X}	0.006 (1, 7.48) ^{X}	0.592 (1, 0.29)	0.567 (1, 0.33)	0.227 (1, 1.46)	0.394 (1, 0.73)	0.883 (1, 0.02)	0.042 (1, 4.13)
NND height class		<0.001 (4, 87.85) *						
Site class x NND height class		0.742 (8, 0.64)						
Height (m)			<0.001 (1, 14.03) *	0.024 (1, 5.1) *	0.533 (1, 0.39)	0.748 (1, 0.1)	0.399 (1, 0.71)	
PFC			0.004 (1, 8.31) *	<0.001 (1, 55.26) *	<0.001 (1, 17.22) ^{X}	<0.001 (1, 25.8) *	0.405 (1, 0.69)	
Year			<0.001 (1, 59.16) ^{X}	0.861 (1, 0.03) ^{X}	0.33 (1, 0.95)	0.321 (1, 0.98) ^{X}	0.088 (1, 2.91)	<0.001 (1, 14.89) *
Height (m) x Year			0.156 (1, 2.01) ^{XX}	0.923 (1, 0.01)	0.778 (1, 0.08) ^{XX}	0.464 (1, 0.54)	0.946 (1, 0)	
PFC x Year			0.1.97(1, 1.16)	0.612 (1, 0.26)	0.029 (1, 4.75)	0.537 (1, 0.38)	0.788 (1, 0.07)	
Site class x Year			<0.001 (2, 19.55) *	0.700 (2, 0.36)	0.906 (2, 0.1)	0.014 (2, 4.26)	0.478 (2, 0.74)	<0.001 (2, 14.12) *
No. of racemes per tree					0.002 (1, 9.83) *	<0.001 (1, 17.83) *	0.278 (1, 1.18)	
Flowering NND					0.79 (1, 0.07)	0.587 (1, 0.29) ^{X}	0.178 (1, 1.82)	
Flowering NND x Year					0.034 (1, 4.47) *	0.868 (1, 0.03)	0.981 (1, 0)	
No. of nuts per ha								<0.001 (1, 10.7) ^{X}
No. of nuts per ha x Year								<0.001 (1, 10.49) ^{X}

4.3.2.2 Effect of Site Attributes on Plant Density

Height class, foreign materials and site class were significantly related to plant density, with compaction and weeds being of lesser importance (Table 4.4). The interaction between site class and height class was not significant. Densities of plants in the three smallest height classes (0-0.5 m, 0.5-1 m and 1-5 m) were significantly greater than those of plants greater than five metres in height (Table 4.5). Plant density was observed to decrease with increasing levels of foreign materials (effect = 0.0065, s.e.d.: 0.0031), and small sites had the highest density of plants (mean NND = 1.44 m), followed by medium (mean NND = 3.51 m) and large sites (mean NND = 7.64 m).

Table 4.5 Table of effects for NND on height class (average s.e.d. = 0.210). Statistically similar height classes for NND are labelled in superscript.

Height Class	*0-0.5 m*	*0.5-1 m*	*1-5 m*	*5-10 m*	*10-50 m*
Effect	0.000 ^{1}	-0.123 ^{1}	0.250 ^{1}	0.693 ^{2}	0.767 ^{2}

4.3.3 Population Dynamics

4.3.3.1 Presence or Absence of Flowering

There was a significant effect of height, PFC, site class, and the interaction between site class and year on the probability of flowering (Table 4.4). Foreign materials, year and height × year were of lesser significance. Height was positively related to flowering (effect = 4.20, s.e.d. = 0.69, Figure 4.3), while the likelihood of flowering decreased as foliage cover increased, with an effect of -4.49 and s.e.d. of 1.39 (Figure 4.4). Plants in small and medium sites were significantly more likely to

flower compared with large sites (Table 4.6). A 24% increase in the proportion of plants flowering was observed in 2003 compared with 2002, with the increase being significantly greater for populations in site class three than in one or two (Figure 4.5).

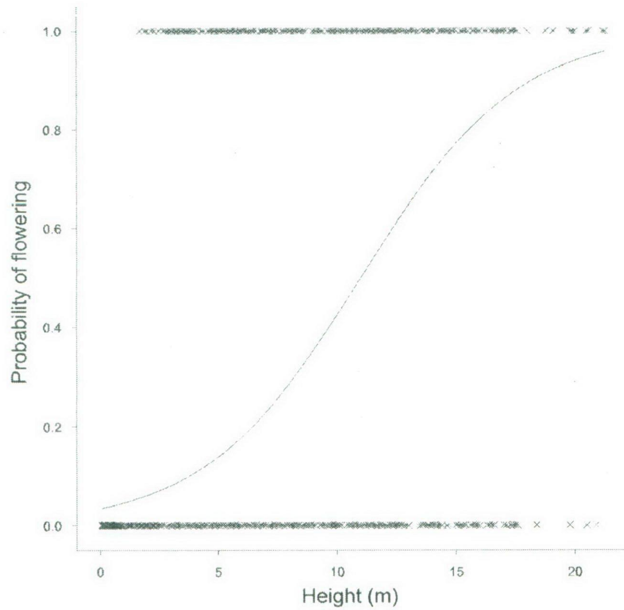


Figure 4.3 Observed and fitted relationships between plant height and probability of flowering across all sites and years. Observed data is shown as crosses, fitted line shows the overall probability of flowering with changes in plant height, based on the observed proportion of plants flowering.

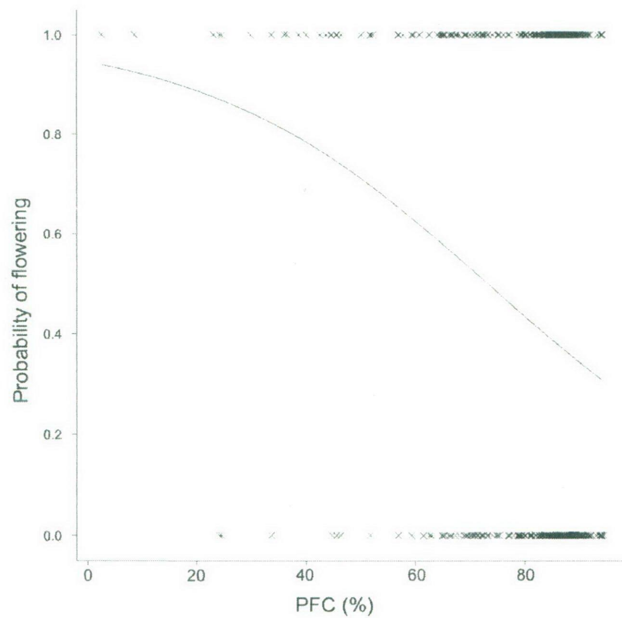


Figure 4.4 Observed and fitted relationship between projected foliage cover (PFC) and probability of flowering across all sites and years. Observed data is shown as crosses, fitted line shows the overall probability of flowering with changes in PFC, based on the observed proportion of plants flowering.

Table 4.6 Table of effects and back-transformed means for proportion of trees flowering in site classes (average s.e.d. = 0.896). Sites that do not differ significantly are labelled in superscript.

Site Class	*Small*	*Medium*	*Large*
Effect	0.000 [1]	0.233 [1]	-3.967 [2]
Back-transformed means	0.313	0.508	0.113

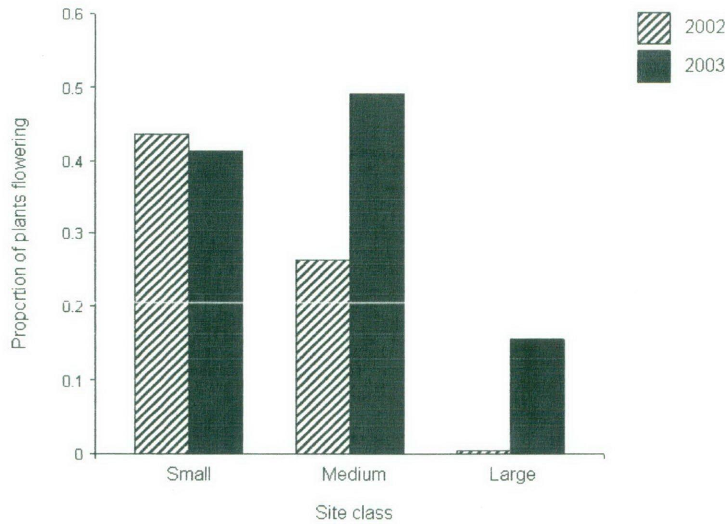


Figure 4.5 Back-transformed mean proportion of plants flowering in site classes in 2002 and 2003.

4.3.3.2 Number of Racemes per Flowering Tree

Computational errors were generated when all terms were included in the model for number of racemes per flowering tree, and were avoided when either foreign materials or weeds were omitted from the analysis. Of these two, weeds explained the least amount of variation (chi pr = 0.567, df = 1, Wald/df = 0.33) and so a final analysis of everything excluding weeds was used. There was a significant effect of height, PFC and foreign materials on the number of racemes produced per flowering tree, while compaction, year and site class were of lesser significance (Table 4.4). The number of racemes per tree increased with increasing plant height (effect = 0.401, s.e.d. = 0.167) and greater levels of foreign materials (effect = 0.006, s.e.d. = 0.002), and decreased as PFC increased (effect = -0.941, s.e.d. = 0.237).

4.3.3.3 Presence or Absence of Fruiting

Probability of flowering plants fruiting was significantly related with number of racemes per tree, flowering NND by year, PFC by year and site class (Table 4.4). PFC, compaction, foreign materials and the interaction between height and year were less important in explaining the variation in the probability of fruiting. Of particular interest are the non-significant relationships between fruiting probability and either plant height or nearest neighbour distance of flowering plants. The probability of fruiting was positively related to number of racemes per tree (effect = 0.00233, s.e.d. = 0.00106), while the relationship between fruiting and the interaction of flowering NND and year was negative between 2002 and 2003 (2003 effect = -0.89, s.e.d. = 0.43). A similar negative relationship between 2002 and 2003 was observed for the interaction between PFC and year (2003 effect = -5.89, s.e.d. = 2.46). A significantly smaller proportion of flowering trees in large sites produced fruit compared with small sites, while medium sites did not statistically differ from either small or large sites (Table 4.7).

Table 4.7 Table of effects and back-transformed means for proportion of flowering trees that produced fruit in site classes (average s.e.d. = 2.245). Sites that do not differ significantly are labelled in superscript.

Site Class	*Small*	*Medium*	*Large*
Regression Coefficient	0.000 ^{1}	-2.973 ^{1,2}	-6.357 ^{2}
Back-transformed means	0.973	0.636	0.078

4.3.3.4 Number of Nuts per Fruiting Tree

Of the terms tested against the number of nuts per fruiting tree, PFC and number of racemes per tree were found to be significant. Flowering NND, foreign materials, year and site class were of less importance (Table 4.4). Plant height was not significantly related to nut production. The number of nuts produced per tree was observed to increase as foliage cover decreased (effect = -0.891, s.e.d. = 0.369), while increasing with the number of racemes per tree (effect = 0.0002, s.e.d. = 0.00005).

4.3.3.5 Number of Nuts per Raceme

There were no significant differences in the mean number of nuts per raceme for any of the terms tested (Table 4.4). Over the two study years, trees in small sites averaged 1.45 nuts per raceme, while medium and large sites had mean values of 1.43 and 1.52, respectively.

4.3.3.6 Recruitment

Site class, year and the interaction between them shared a significant relationship with number of recruits per hectare (Table 4.4). These factors appeared to capture most of the variation in recruitment, although foreign materials, nuts produced per hectare and the interaction between nuts per hectare and year were closely aligned with one or more levels of these factors. Recruitment in small sites was significantly greater than in medium or large sites across both years (Table 4.8), however the number of new seedlings in small sites was substantially higher in 2003 than 2004 (Figure 4.6). No difference was observed between years for the other site classes.

Table 4.8 Mean number and s.e.m. of new seedlings per hectare per year for small, medium and large site classes in 2003 and 2004.

Site Class	*Mean*	*s.e.m.*
small	6.85	2.89
medium	0.33	0.16
large	0.21	0.02

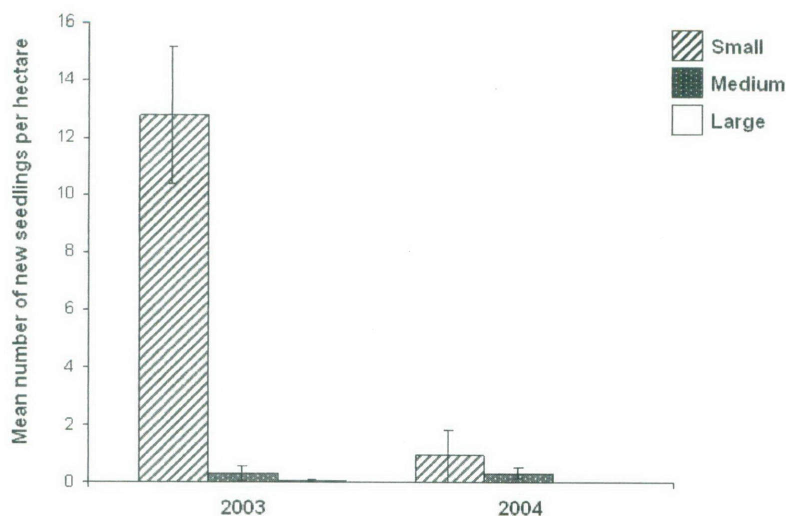


Figure 4.6 Mean recruitment per hectare (± s.e.m.) by site class and year.

4.3.3.7 Mortality

Due to the small number of plant deaths observed during the course of this study, statistical analysis of results was not attempted. Mortality in each site is instead summarised in Table 4.9. 59% of deaths were seedlings underneath one isolated maternal tree in site 76, with smothering by leaf litter appearing to be the likely cause. These deaths are regarded as an outlier, however, and without them there is little evidence to suggest that mortality is different between sites and site classes. The

majority of deaths overall were of small plants under 0.3 m, with only two deaths of plants above 10 m observed. One of these trees was crushed by falling debris during a severe storm in 2002, while the other died from unknown causes.

Table 4.9 Observed number of *M. integrifolia* deaths in study sites in the intervals between 2002 and 2003, and 2003 and 2004.

Site class	*Site*	*Total no. of individuals in sampled area*	*No. of deaths*	
			2002-2003	*2003-2004*
Small	8	113	0	1
	70	135	0	2
	76	96	18	38
Medium	20	176	2	4
	77	115	8	1
	120	94	0	0
	134	249	0	0
Large	128	233	0	0
	129	235	0	0
	131	284	0	0

4.4 Discussion

The findings from this study suggest that habitat fragmentation is not detrimentally affecting population demography of *M. integrifolia*, and that reproductive output and recruitment in the smallest sites are actually exceeding that in the larger sites with no apparent difference in mortality rates. Height class distributions for the three site classes all possessed an inverse J-shape, a characteristic commonly observed for rainforest tree species and often associated with healthy regeneration patterns (Poorter et al. 1996). Trees in small sites were found to have a higher probability of flowering and fruiting than those in large sites, despite being shorter on average and occurring at higher densities. Numbers of racemes per flowering tree, nuts per fruiting tree, and nuts per raceme were not observed to differ between the three site classes.

Therefore while fecundity at the tree and raceme level did not appear to be affected by fragmentation, reproductive output at the site level is enhanced in small sites through a higher proportion of individuals reproducing. Recruitment levels in small sites were greater than in large sites, while no evidence could be found to suggest that mortality levels differed significantly, suggesting that population growth is occurring at a greater rate in small sites compared with large sites. The length of census for this study (three years) is relatively short, however, and longer-term census work is required to test this more conclusively. Medium sites were observed to fall between small and large sites for many of the parameters tested, including height and plant density. Similar proportions of trees flowered in medium and small sites, and while small and large sites differed significantly in the proportion of flowering trees that produced fruit, medium sites were not statistically different from either. Recruitment levels in medium sites were similar to those in large sites.

4.4.1 Demographic State

The demographic structure of a population reflects its recruitment and mortality history (Harper 1977), and can be indicative of its demographic future (Bruna & Kress 2002). Despite its relationship with population viability, demographic structure has been examined in very few fragmentation studies to date. In this project, height class distributions for all three site classes suggested healthy regeneration patterns, however those for small and medium sites were shifted more toward smaller size classes, with proportionally fewer larger plants, and greater numbers of small individuals than in the large site classes. A similar pattern was observed by Bruna and Kress (2002) in fragmented and continuous populations of *Heliconia acuminate*

(Heliconiaceae), an understorey shrub native to Amazonian tropical forests. In their study approximately 20% of individuals in continuous forest fell into the two largest size classes, compared with only 15% in 10 ha fragments and 8% in one-hectare fragments. Several non-mutually exclusive mechanisms are listed by these authors as being potentially responsible for such findings: (i) increased mortality of larger individuals following fragmentation; (ii) a flush of post-fragmentation recruitment in forest fragments; and (iii) the shifting of surviving individuals from large to smaller size classes (via plant die-back, physical damage, etc.). The higher recruitment and stable mortality levels seen in small compared with large *M. integrifolia* sites in this study suggests that the second of these mechanisms may be partially responsible for the observations described above. While prior mortality of larger individuals and/or the shifting of existing taller trees to shorter cannot be disregarded in fragmented populations of *M. integrifolia*, distinct differences in growth habit were also observed between trees in small and large sites, allowing a potential fourth mechanism for this species: changes in plant habit between fragmented and non-fragmented sites. Mature *M. integrifolia* trees in small sites often physically resembled cultivated trees of the species more closely than those in large sites, being shorter on average, with wider trunk and branch girths and more developed canopies. Given their similarity to cultivated plants, this change in habit could be attributed to increased resource availability, in particular light availability.

In a study into the Brazil nut tree (*Bertholletia excelsa*, Lecythidaceae), Peres et al. (2003) observed that natural populations that had been persistently and intensively harvested for nuts lacked juvenile plants under 60 cm diameter at breast height, threatening long-term population survival. In *M. integrifolia*, very little harvesting

from natural populations was observed during the course of this study, which in addition to the healthy regeneration patterns found, suggests that threat from harvesting is likely to be negligible.

4.4.2 Population Dynamics

Fragmentation theory predicts that the reduction and fragmentation of forests is likely to have negative consequences for reproductive success (Cascante et al. 2002; Cunningham 2000b; Heywood et al. 1994; Laurance et al. 1998a; Laurance et al. 1998b; Whitmore & Sayer 1992), recruitment (Benítez-Malvido & Martinez-Ramos 2003; Bond 1995; Scariot 1999; Tabarelli et al. 1999) and mortality (Laurance et al. 1998a) of tree species. Most empirical studies into the impact of fragmentation on population dynamics, however, have predominantly focussed on pollination and fruit and seed set at the scale of the individual, and to a lesser extent, recruitment and mortality. Very few papers have assessed flowering except in a pollination context, and only two studies could be found that investigated changes in flowering at the site level with fragmentation (Bruna & Kress 2002; Pisanu 2001). The finding that a greater proportion of plants flowered in small and medium *M. integrifolia* sites compared with large sites contrasts with that by Bruna and Kress (2002) in fragmented *H. acuminata* populations. In their study, Bruna and Kress observed marginally significant differences in proportion of flowering between site classes in 1998 and no significant differences in 1999, although there was a trend in both years towards a greater proportion of plants flowering in continuous forest sites. In the subtropical rainforest tree *M. tetraphylla*, Pisanu (2001) observed no relationship between the total number of flowers or seeds produced and either population size or

level of disturbance. No other studies could be found that investigated fragmentation impacts on fruiting at the site level. Similar to the results observed for flowering in this study, the proportion of flowering *M. integrifolia* individuals that subsequently produced nuts in small sites was significantly greater in small than large sites, while medium sites did not differ significantly from either. Given the physical similarity of trees in small sites to cultivated plants, these findings could be attributed at least partially to increased resource availability in these sites, however the absence of any differences in flowering and fruiting at the individual level between site classes suggests that other factors may be involved.

Empirical studies into the fecundity of trees in fragmented populations compared with continuous habitat have found evidence of reductions (Aizen & Feinsinger 1994a; Cascante et al. 2002; Cunningham 2000b; Ghazoul et al. 1998; Nason & Hamrick 1997), increases (Aizen & Feinsinger 1994a; Dick 2001), and no detectable change (Bruna & Kress 2002). The latter of these was observed for both number of flowers per flowering tree and number of nuts per fruiting *M. integrifolia* tree in this study. Reproductive success of plants is determined by: (i) pollen availability for fertilisation (governed by pollinator activity and pollen quantity and quality); and (ii) available resources for seed production, e.g. light, water and nutrients (Aizen & Feinsinger 1994a; Nason & Hamrick 1997; Zimmerman & Pyke 1988). Given the apparent stability in fecundity between the different site areas, it may be the case that pollen and resource availability are equal between site classes, or that a limitation in one for any particular site class is offset by an increase in the other.

Similar to the seeds of many rainforest tree species (Vázquez-Yanes & Orozco-Segovia 1993), *M. integrifolia* nuts are not capable of extended storage in the canopy or soil seed bank (Hamilton 1957). Regeneration of the species often depends therefore on the ability of seeds to germinate beneath an often intact canopy and persist as seedlings or juveniles for extended periods (Nadolny 1999). In this study, recruitment in small sites was significantly greater than in medium and large sites. Germination was not statistically related to the number of nuts produced per hectare in the previous season, and so the increased recruitment observed is more likely to be associated with environmental or biological factors (Nadolny 1999; Vázquez-Yanes & Orozco-Segovia 1993). Conditions in small forest fragments are typically hotter and drier, with increased light penetration compared to continuous forest (Kapos et al. 1997). These factors can all affect the cues required for germination, however they are more commonly associated with reduced germination in fragments (Bruna 1999; Nason & Hamrick 1997). Another possible explanation may be differences in nut predation between site classes (Curran & Leighton 2000).

Seedling survivorship in fragmented habitats can be detrimentally affected by many factors including increased herbivory (Benítez-Malvido 2002), disturbance (Benítez-Malvido 1998), competition (Sizer & Tanner 1999), litterfall (Bruna 1999, 2002; Janzen 1983; Sizer et al. 2000), fungal infection (Benítez-Malvido et al. 1999), and damage from falling debris (Scariot 2001). Observed mortality of *M. integrifolia* in this study was very low across all site classes, similar to results observed by Nason and Hamrick (1997). In their study, seedling mortality of *Spondias mombin* (Anacardiaceae), a canopy tree common to forests in tropical Central America, was

found to be low across all habitat areas, despite significant differences in germination rates between study sites.

4.4.3 Conclusions

While the above findings suggest that population demography and demographic processes in *M. integrifolia* have indeed been influenced by habitat fragmentation, these changes may not necessarily negatively impact population viability. Conversely, population growth appears to be more strongly positive in small fragments compared with medium and large sites, at least in the short-term, resulting from increased site-level fecundity and recruitment, and no apparent change in short-term mortality. Population viability may also benefit from the long life span of the species and observed resilience of adult plants to disturbance, potentially buffering populations against stochastic events. Resource availability, in particular increased light levels in fragments, is raised here as a potentially important factor in determining the demography of *M. integrifolia* populations, and investigations as to its significance are worthwhile for future studies. Longer-term estimation of population viability requires knowledge of fragmentation impacts on mating system and other population genetic processes, and is investigated in Chapters 4 and 5. Similar to the findings of Bruna and Kress (2002), the results observed here highlight the importance of assessing demographic changes at the site level. The majority of studies into the demography of fragmented populations focus primarily on fecundity at the individual level only, which as demonstrated here, can overlook potentially critical population-level variation.