CHAPTER V:

Male Population Structure and Dispersal in *Macropus fuliginosus* and Hybridisation with *Macropus giganteus* as Revealed by Y-microsatellite Haplotypes

Abstract

There are several aspects of biology in which the contribution of males and females is unequal. In these instances the examination of Y chromosome markers may be used to elucidate male specific attributes. Herein male dispersal patterns and genetic structuring were examined at four Y-microsatellite loci in 183 male western grey kangaroos (*Macropus fuliginosus*) from throughout the species' trans-continental distribution. In addition, a sample of both *M. fuliginosus* and the eastern grey kangaroo (*Macropus giganteus*) from their region of sympatry, were examined to determine the male contribution in hybridisation. In general, diversity across Y-haplotypes was low, potentially resulting from the skewed sex ratio and polygynous mating system. As expected, male dispersal was high across the range. However, the Lake Torrens/ Flinders Ranges region evidently restricts the dispersal of male *M. fuliginosus*. This suggests that the admixture evident at other mainland genetic group boundaries may be associated with high male dispersal. Four *M. giganteus* individuals were found to possess introgressed *M. fuliginosus* Y-haplotypes, further revealing the rare hybridisation events which naturally occur between species of grey kangaroo. Examination of the Y-chromosome and elucidation of male specific traits is an important aspect of molecular ecology as it provides a means of differentiating male specific attributes of population history, structure and hybridisation.

Introduction

For aspects of ecology, parentage and migration the contribution of the sexes is often unequal. Mammalian species are typically characterised by female philopatry and male dispersal (Greenwood, 1980; Johnson, 1989). Thus males and females do not contribute symmetrically to population genetic structure. Despite this, Y-linked markers have only been used relatively rarely compared to maternally and bi-parentally inherited markers. However, an examination of the Y-chromosome is increasingly being employed in genetic studies, allowing the investigation of dispersal, mating systems and other sex-specific population parameters (Hurles and Jobling, 2001; Petit *et al.*, 2002).

Previously male specific parameters were deduced via comparisons of bi-parentally and maternally inherited markers (Firestone *et al.*, 1999; Hoarau *et al.*, 2004; Pope *et al.*, 2000; Wilmer *et al.*, 1999; Zenger *et al.*, 2003a). However, direct examination of variation on the Y-chromosome provides an independent source of information on the contribution of males to reproduction and genetic structure (Hurles and Jobling, 2001). In humans, variation at the Y-chromosome has revealed the origins (Hammer *et al.*, 1998), population histories (Karafet *et al.*, 1999), sex biased admixture (Hurles *et al.*, 1998) and differences in migration routes and rates between males and females (Hurles *et al.*, 1999; Seielstad *et al.*, 1999). Furthermore, Y-linked markers are being increasingly applied to other species to infer male specific structuring and histories in baboons (Lawson Handley *et al.*, 2006), shrews (Handley and Perrin, 2006), wolves (Sundqvist *et al.*, 2001) and cattle (Hanotte *et al.*, 2000), as well as biases in hybridisation (Ward *et al.*, 2001). Y-linked markers provide information on male specific attributes which were not evident by comparison between markers. In the western grey kangaroo *Macropus fuliginosus,* examination at both maternally (Chapter III) and bi-parentally (Chapter II) inherited loci have left gaps in regard to male movements and population histories, which may be elucidated via recently available Y-linked microsatellite makers (Macdonald *et al.*, 2006).

Initially discovered on Kangaroo Island in 1802 (Flinders, 1814), the distribution of *M. fuliginosus* stretches continuously across much of southern Australia, from the

west coast to almost the Great Dividing Range in the east, reaching as far north as Shark Bay in the west and into south-western Queensland in the east (see Figure V-1). The distributional limits are dictated by winter dominated annual rainfall, greater than 200mm (Cairns *et al.*, 1991; Caughley *et al.*, 1987). Typically *M. fuliginosus* inhabits heath, while moving onto open grassland to feed (Coulson, 1990). Currently two subspecies are recognised, an insular subspecies, *M. fuliginosus fuliginosus* on Kangaroo Island, South Australia, and the mainland subspecies, *M. fuliginosus melanops* (Kirsch and Poole, 1972), which are distinguished by significant differences in morphology (Poole *et al.*, 1990) and several reproductive traits (Poole, 1975; Poole, 1976; Poole and Catling, 1974). However, this subspecific classification appears unwarranted based on genetic analyses (see Chapters II & III).

High levels of male migration in *M. fuliginosus* have been inferred from comparisons of maternally and bi-parentally inherited markers (Chapter III). However, no significant differences between the relatedness and assignment probabilities of males and females were evident (Chapter II). Examination of the Y-chromosome may aid in further elucidating the extent of male dispersal. Furthermore, previous studies have revealed five distinct genetic units, which exist within geographically circumscribed regions of the distribution including Kangaroo Island, eastern, central, north-west and south-western Australia (Chapter II). Of these regions the division in the north-western and south-western regions of the range were apparent at both maternally and bi-parentally inherited markers. Conversely most of the current eastern range of *M. fuliginosus* was reached in a single expansion event in the late Pleistocene and hence the remaining mainland barriers to distribution were only evident in the microsatellite data (Chapters II & III). Examination of paternally inherited markers will provide further clarification of these boundaries, indicating the extent to which the free movement of males, the dispersing sex is impeded.

Consideration of the partitioning of Y-chromosome genetic variation may also reveal the colonisation histories of various populations. As a continental island, Kangaroo Island formed part of the mainland prior to its isolation, approximately 9,500 years ago (Thom and Chappel, 1975). The events resulting in the separation

of Kangaroo Island probably began approximately 12,000 – 13,500 years ago but the island remained connected to the mainland via two land-bridges from Fleurieu and Yorke Peninsulas until 9,300 – 10,500 and 8,800 – 9,900 respectively (Thom and Chappel, 1975). The absence of historic bottlenecks in the population indicated that colonisation took place prior to the initiation of these events since, presumably during the eastern expansion on the mainland, approximately 60,000 years ago (Chapter III). The mtDNA sequence data also revealed that the female lineage was derived from *M. fuliginosus* on the eastern mainland (east of the Flinders Ranges). However, in several respects *M. fuliginosus* on Kangaroo Island are more similar to those of the western mainland (Clark and Poole, 1973; Mead, 1985b; Poole and Catling, 1974). Moreover, a potential route between Western Australia and Kangaroo Island appeared during the last glacial phase, when the continental shelf of southern Australia was exposed (Bowler, 1982; Kershaw *et al.*, 2003). Drift may have resulted in many of the similarities but examination of the paternal lineage may further elucidate the origins of all *M. fuliginosus* on Kangaroo Island.

Parentage and mating systems represent another facet in which the sexes may not contribute equally. Hybridisation between closely related species has been shown to be asymmetrical in several species (Ward *et al.*, 2001), with introgression of mtDNA shown to extent past hybrid zones and into the wider populus in some instances (Goodman *et al.*, 1999; Ruedi *et al.*, 1997). However, only relatively few examples have examined the differential inheritance of uni-parentally inherited markers (Ward *et al.*, 2001). *M. fuliginosus* shares most of its eastern range with its sister species, *M. giganteus* (Caughley, 1984). Recently evidence of introgression was identified between these two species within their region of sympatry (Chapter IV). The rates of hybridisation however, are low and secondary contact probably only occurred during the Pleistocene, when *M. fuliginosus* invaded the eastern regions of its range (Chapter III). Under captive conditions, hybridisation between the grey kangaroos appeared uni-directional, resulting from *M. fuliginosus* males and *M. giganteus* females, with the reciprocal cross never observed (Kirsch and Poole, 1972; Poole and Catling, 1974). Furthermore, male *M. fuliginosus* were also more likely to mate with hybrid females compared with male *M. giganteus*. Conversely introgression of microsatellite alleles was found to occur in both directions in the region of sympatry indicating successful hybridisation in both

directions may be possible (Chapter IV). However, hybrids appeared to more frequently be associated with the *M. giganteus* maternal lineage. Therefore the investigation of Y-haplotypes may further elucidate the extent of introgression between the two species and identify potential reasons for its occurrence.

Recently, Y-linked macropod microsatellite markers were isolated from the tammar wallaby, *Macropus eugenii* and successfully amplified in other macropods including the closely related eastern grey kangaroo, *Macropus giganteus* (Macdonald *et al.*, 2006). Herein we examine the genetic structure of *M. fuliginosus* from 20 populations across the range at Y-chromosome microsatellite loci. Furthermore both species of grey kangaroo in the region of sympatry were examined for evidence of introgression of Y-haplotypes, focussing on previously identified hybrids.

Materials and Methods

Sample Collection and PCR Amplification

180 male *M. fuliginosus* were sampled from 20 populations across the distribution, including one population of the insular subspecies, *M. f. fuliginosus* from Kangaroo Island, South Australia (Figure V-1). Sample collection has previously been described in Chapter II. Briefly, tissue samples were solicited from commercial kangaroo harvesters, government agencies involved in control measures and researchers. Typically approximately 10 individuals were sampled from each site which may have included several social groups (see Table V-1). In addition, extra samples were obtained from Byrock (n=1), Glenariff (n=1) and Kersbrook (n=1). In order to investigate potential introgression of Y-haplotypes in grey kangaroos, 17 *M. giganteus* and 10 backcrossed (5 *M. giganteus* and 5 *M. fuliginosus*; see Chapter IV) individuals from the within the region of sympatric were tested in addition to the 25 *M. fuliginosus* already sampled. Samples were skin, liver or kidney and were preserved in 80% ethanol, a 20% DMSO $NaCl_2$ solution (Kilpatrick, 2002) or frozen at -20°C in the field. DNA extraction was carried out according to Sunnucks and Hale (1996) or Sigg *et al.* (2005).

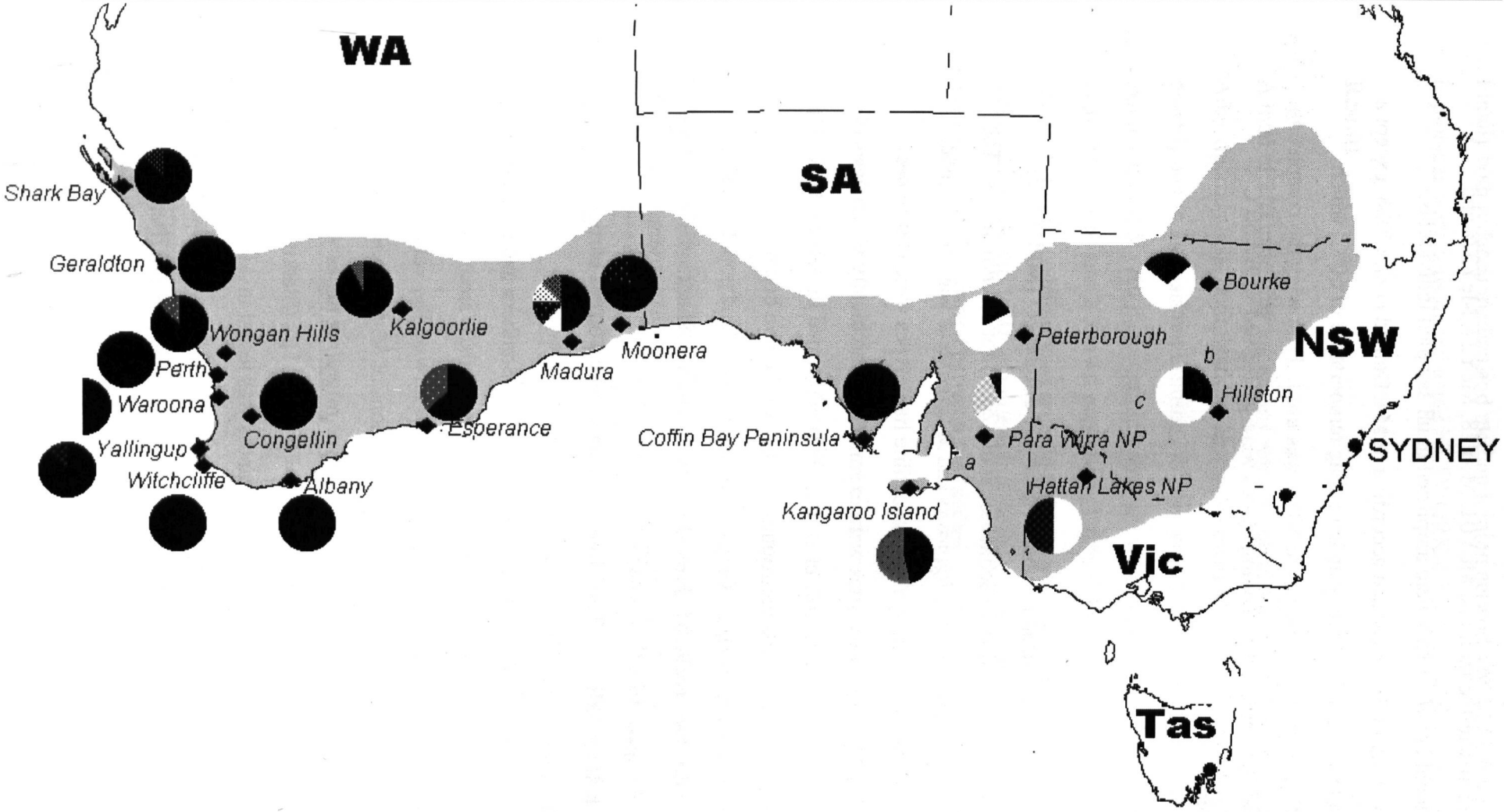

Figure V-1: The distribution of *Macropus fuliginosus* (shaded grey), showing the location of sampled sites (a. Kersbrook (n=1), b. Glenariff (n-1) and c. Byrock (n=1)) and the proportion of Y chromosome haplotypes identified within.
Haplotype A B C D E F G H I J K L

Five Y-linked microsatellite markers, MeY01, MeY28, MeY27, MeY37a and MeY37b (Macdonald *et al.*, 2006) were amplified in all sampled individuals. Amplification was via PCR, performed according to MacDonald *et al.* (2006) except a 'touchdown' cycle was used, whereby an annealing temperature of 60°C, which decreased in 2°C increments each cycle to 50°C, was used. Following the completion of the last touchdown cycle the remaining cycles were performed with a 50°C annealing temperature.

Analysis

Haplotypic diversity and the mean number of pairwise differences (MPD) were estimated in ARLEQUIN version 2 (Schneider *et al.*, 2000) for *M. fuliginosus*. The level of differentiation between *M. fuliginosus* and *M. giganteus* was also calculated in ARLEQUIN. To determine how Y-microsatellite haplotypes were partitioned across the range of *M. fuliginosus*, sites were examined hierarchically via analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) performed in ARLEQUIN. The analysis was conducted to test the partitioning at each of the five boundaries previously identified (Chapter II) i. Kangaroo Island vs. mainland populations; ii. The Nullarbor Plain (Kalgoorlie/Madura/Moonera vs. Albany/Shark Bay/Geraldton/Wongan Hills/Waroona/Congelin/Esperance/ Yallingup/Witchcliffe/Perth) iii. The Flinders Ranges/Lake Torrens barrier (Kalgoorlie/Madura/Moonera/Coffin Bay Peninsula vs. Hillston/Bourke/Peterborough/ParaWirra) and finally iv. Southern and northern Western Australia (Perth/Shark Bay/Geraldton/Wongan Hills/Waroona vs. Congelin/Esperance/ Yallingup/Witchcliffe/Albany). Pairwise R_{ST} values and associated p-values were calculated between populations of *M. fuliginosus* in ARLEQUIN. An estimate of the number of effective migrating males was calculated from the R_{ST} values using the relationship $N_e m_m = [(1/Rst)-1]/2$ (Slatkin, 1993). The correlation between genetic and geographic distances was tested in ARLEQUIN using Mantel's test. The correlation was examined overall as well as within eastern (Coffin Bay Peninsula/Hillston/Bourke/Peterborough/Para Wirra) and western (Perth/Shark Bay/Geraldton/Wongan Hills/Waroona vs. Congelin/Esperance/ Yallingup/Witchcliffe/Albany/ Kalgoorlie/Madura/Moonera) regions of the range.

Finally both reduced median and median joining networks of all *M. fuliginosus* haplotypes were constructed using NETWORK 4.2.0.1 (Bandelt *et al.*, 1999).

Results

A total of 210 male grey kangaroos were genotyped at 5 Y-linked microsatellite loci. After rigorous PCR optimization (data not shown) MeY27 could not be scored reliably due to inconsistent banding patterns and allelic drops out and so was not examined further. All the remaining markers were polymorphic displaying 3-5 alleles in *M. fuliginosus* and 2-5 in *M. giganteus* (Table V-1). Among the 183 *M. fuliginosus* sampled, 13 unique haplotypes were identified, while 4 unique haplotypes were identified within the 16 *M. giganteus* individuals examined (Table V-2). An average R_{ST} value of 0.75 occurred between the species.

Table V-1: Characteristics of four Y-linked microsatellites in grey kangaroos, *Macropus fuliginosus* (n=138) and *M. giganteus* (n=16).

Microsatellite	Number of Alleles		Allele Range (bp)	
	M. giganteus	*M. fuliginosus*	*M. giganteus*	*M. fuliginosus*
MeY01	2	3	242-245	245-340
MeY28	2	5	362-365	336-350
MeY37a	2	4	168-170	170-176
MeY37b	3	4	168-172	172-182

Of the 13 haplotypes found within the range of *M. fuliginosus*, A and B were the most common, found in 155 of the 184 individuals sampled (Table V-3), and only differed by one repeat at loci MeY37a (Table V-2; Figure V-2). The A haplotype was found predominately in populations in the west of the range, while haplotype B was found in the east. Of the remaining mainland haplotypes E, H and K were found in the west, F, G, I and J in the Nullarbor region (Kalgoorlie, Madura and Moonera) and L, H and C in the east (Figure V-1). Two haplotypes were located on Kangaroo Island, haplotype A, found on the mainland and D, which was specific to the island, occurred in an approximately equal ratio (Figure V-1; Table V-3). D differed from A by one repeat at both MeY37a and MeY37b (Table V-2).

Table V-2: Y-chromosome haplotypes and allele sizes (in base pairs) at four microsatellite loci found in grey kangaroos. Haplotypes A – L occurred across the distribution of the *Macropus fuliginosus*, while M – P were found in *Macropus giganteus*.

Haplotype	MeY01	MeY28	MeY37a	MeY37b
A	245	342	172	174
B	245	342	174	174
C	245	342	172	176
D	245	342	170	172
E	245	338	172	174
F	245	342	172	172
G	245	342	170	174
H	245	342	174	176
I	245	344	172	174
J	245	336	172	174
K	340	350	176	182
L	307	342	174	174
M	245	365	168	170
N	245	365	170	170
O	245	365	170	172
P	242	362	168	168

Table V-3: Distribution of Y chromosome haplotypes in populations sampled throughout the distribution of *Macropus fuliginosus*. Sample sizes are shown in brackets.

	A	B	C	D	E	F	G	H	I	J	K	L
Kangaroo Island (13)	6			7								
Albany (3)	3											
Yallingup (8)	7							1				
Witchcliffe (3)	3											
Shark Bay (7)	6										1	
Geraldton (9)	9											
Perth (10)	10											
Wongan Hills (16)	14				2							
Congelin (9)	9											
Esperance (14)	9				5							
Kalgoorlie (14)	13					1						
Madura (9)	4	1					2		1	1		
Moonera (5)	4						1					
Coffin Bay (15)	15											
Peterborough (6)	1	5										
Para Wirra NP (15)		10	4					1				
Hattah Lakes NP (2)		1						1				
Hillston (14)	4	10										
Bourke (7)	1	5										1
Kersbrook (1)			1									
Byrock (1)		1										
Glenariff (1)		1										
Waroona (2)	1	1										

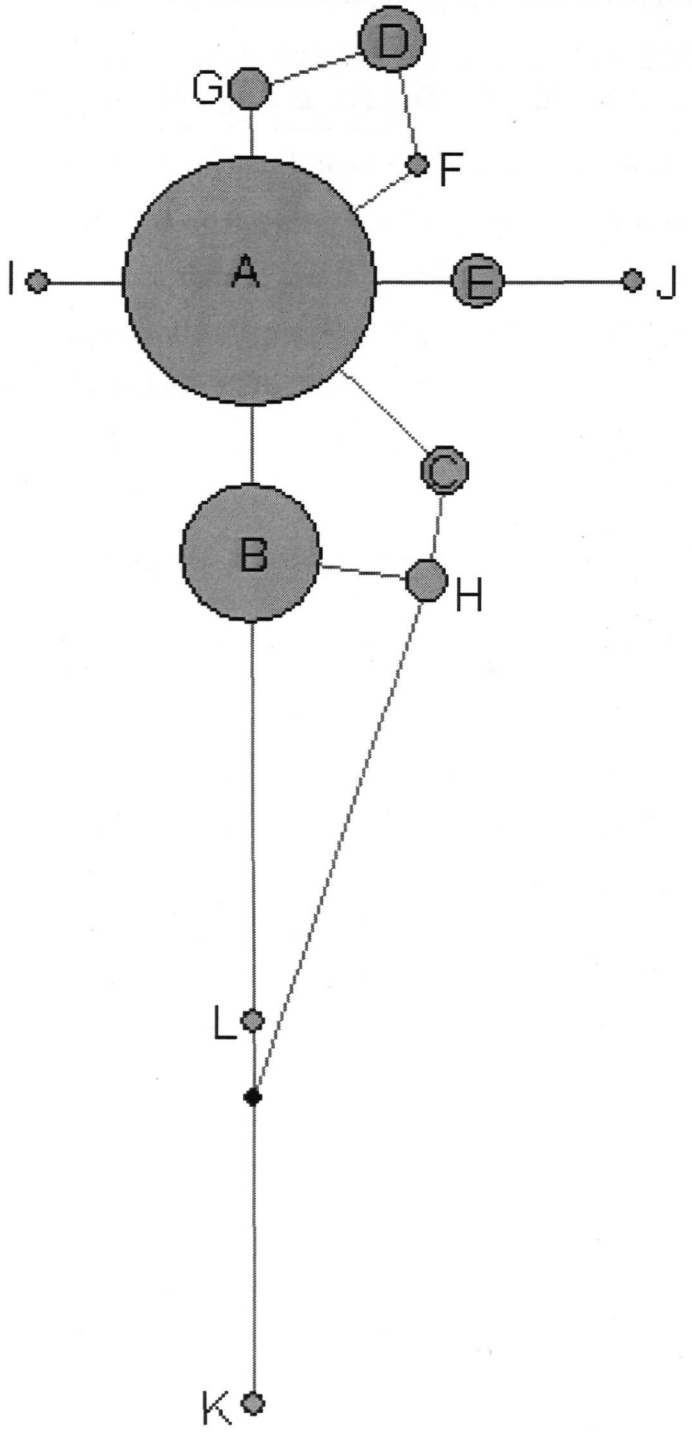

Figure V-2: Reduced median network of Y chromosome microsatellite haplotypes across the distribution of *M. fuliginosus*. Circles represent sampled haplotypes and the size represents the relative frequency. Solid circle indicates a median vector. A single mutation point separates connected haplotypes except for B and L as well as H and the median vector which are separated by 32 mutation points, while 28 mutation points occur between median vector and K.

In general there was little diversity displayed in *M. fuliginosus* populations with only 0.07 haplotypes found per sample analysed. In 6 sites, Albany, Witchcliffe, Congelin, Geraldton, Perth and Coffin Bay only one haplotype was located, despite relatively extensive sampling at some sites (n>9; see Table V-4). Madura possessed the greatest diversity with 5 haplotypes located in the 9 sampled individuals.

Table V-4: Genetic diversity in Y microsatellite haplotypes in *Macropus fuliginosus*, including haplotypic diversity and mean pairwise differences (MPD).

Locality	Sample size	Number of haplotypes	Haplotypic diversity (±SD)	MPD (±SD)
Kangaroo Island	13	2	0.538 (±0.06)	1.077 (±0.76)
Albany	3	1	-	-
Yallingup	8	2	0.250 (±0.18)	0.500 (±0.47)
Witchcliffe	3	1	-	-
Shark Bay	7	2	0.286 (±0.20)	1.143 (±0.84)
Geraldton	9	1	-	-
Perth	10	1	-	-
Waroona	2	2	1.000 (±0.50)	1.000 (±1.00)
Wongan Hills	16	2	0.233 (±0.13)	0.233 (±0.29)
Congelin	9	1	-	-
Esperance	14	2	0.495 (±0.09)	0.494 (±0.45)
Kalgoorlie	14	2	0.133 (±0.11)	0.133 (±0.21)
Madura	9	5	0.806 (±0.12)	0.972 (±0.72)
Moonera	5	2	0.400 (±0.24)	0.400 (±0.44)
Coffin Bay	15	1	-	-
Peterborough	6	2	0.333 (±0.21)	0.333 (±0.38)
Para Wirra NP	15	3	0.514 (±0.12)	0.895 (±0.66)
Hattah Lakes NP	2	2	1.000 (±0.50)	1.000 (±1.00)
Hillston	14	2	0.440 (±0.11)	0.440 (±0.42)
Bourke	7	3	0.524 (±0.21)	0.571 (±0.52)

Pairwise comparisons between sampled localities revealed that relatively few sites displayed significant differences and those which did were typically separated by large distances (>1000km), Perth and Peterborough for example (see Table V-5). Significant levels of differentiation were also evident between Kangaroo Island and mainland populations. Hierarchical analysis of sites via AMOVA revealed that significant partitioning of haplotypes was only evident across the Flinders Ranges/Lake Torrens barrier ($p<0.05$), with 57.24% of the variation partitioned between the two groups and 39.46% within populations. Only 31% was accounted for between Kangaroo Island and the mainland.

A positive correlation with geographic distance ($p=0.001$; Figure V-3) was apparent, with 46% of the variation explained. However, the relationship was not evident when either the eastern ($p=0.25$) or western ($p=0.40$) regions were examined alone. High levels of male migration were apparent, with many sites indicating an infinite number of male migrants (Table V-5).

Within the region of sympatry 52 grey kangaroos were examined, 25 had previously been identified as *M. fuliginosus*, 17 as *M. giganteus* and 5 of each backcross to the respective species. All the individuals examined, except 4 possessed a Y-haplotype consistent with the previously assigned species. Among those individuals previously identified as backcrosses, all individuals possessed a haplotype consistent to the species to which they were backcrossed, including individual 43, which displayed introgression of mtDNA. The remaining 4 individuals were previously identified as *M. giganteus* but displayed introgressed *M. fuliginosus* Y haplotypes. All of these individuals possessed haplotype A and upon examination of microsatellite variation, 3 of the 4 individuals exhibited 1-2 potentially introgessed alleles at the 12 loci (Table V-6).

Table V-5: Levels of population differentiation at the Y haplotypes in *Macropus fuliginosus*. R_{ST} values are above the diagonal and migration rates ($N_e m_m$) below. Inf. = Infinite. * denotes significance $p<0.05$.

	Kangaroo Island	Albany	Congelin	Witchcliffe	Yallingup	Perth	Waroona	Wongan Hills	Geraldton	Shark Bay	Esperance	Kalgoorlie	Madura	Moonera	Coffin Bay	Peterborough	ParaWirra NP	Hattah Lakes NP	Bourke	Hillston
Kangaroo Island		0.30	0.45*	0.30	0.48*	0.46*	0.50	0.36*	0.45*	0.10*	0.39*	0.45*	0.15	0.23	0.53*	0.71*	0.70*	0.75*	0.11*	0.71*
Albany	1.18		0.00	0.00	-0.17	0.00	0.25	-0.14	0.00	-0.17	0.09	-0.19	-0.25	-0.13	0.00	0.73*	0.39*	0.77	-0.16	0.53*
Congelin	0.62	Inf.		0.00	0.02	0.00	0.66	0.02	0.00	0.04	0.25	-0.04	-0.08	0.13	0.00	0.84*	0.51*	0.92*	0.04*	0.64*
Witchcliffe	1.18	Inf.	Inf.		-0.17	0.00	0.25	-0.14	0.00	-0.17	0.09	-0.19	-0.25	-0.13	0.00	0.73	0.39*	0.77	-0.16	0.53
Yallingup	0.54	Inf.	31.51	Inf.		0.03	-0.04	0.02	0.02	0.02	0.21	0.10	-0.06	0.09	0.09	0.51*	0.31*	0.56	0.02*	0.41*
Perth	0.59	Inf.	Inf.	Inf.	16.47		0.69	0.03	0.00	0.05	0.26	-0.03	-0.07	0.15	0.00	0.85*	0.53*	0.92	0.06*	0.65*
Waroona	0.51	1.50	0.26	1.50	Inf.	0.23		0.05	0.66	-0.31	0.10	0.51	-0.19	0.37	0.79	-0.09	-0.11	0.00	-0.31	-0.23
Wongan Hills	0.87	Inf.	31.51	Inf.	20.74	19.31	9.60		0.02	0.13	0.08	0.05	-0.09	-0.01	0.07	0.46*	0.42*	0.53*	0.13*	0.43*
Geraldton	0.62	Inf.	Inf.	Inf.	31.51	Inf.	0.26	31.51		0.04	0.25	-0.04	-0.08	0.13	0.00	0.84*	0.51*	0.92*	0.04*	0.64*
Shark Bay	4.53	Inf.	12.60	Inf.	24.87	8.75	Inf.	3.37	12.60		0.11*	0.12	0.04	-0.05	0.13	-0.02	0.12	-0.31	-0.15	0.11
Esperance	0.79	5.25	1.54	5.25	1.84	1.43	4.44	5.88	1.54	4.04		0.30*	0.01	0.17	0.33*	0.40*	0.43*	0.39	0.11*	0.44*
Kalgoorlie	0.60	Inf.	Inf.	Inf.	4.58	Inf.	0.48	8.65	Inf.	3.74	1.15		-0.04	0.09	0.00	0.78*	0.56*	0.87*	0.12*	0.64*
Madura	2.75	Inf.	Inf.	Inf.	Inf.	Inf.	Inf.	Inf.	Inf.	12.51	34.89	Inf.		-0.15	-0.03	0.24*	0.30*	0.19	0.04*	0.29*
Moonera	1.63	Inf.	3.46	Inf.	5.05	2.86	0.84	Inf.	3.46	Inf.	2.42	5.13	Inf.		0.26	0.73*	0.53*	0.74*	-0.05	0.64*
Coffin Bay	0.44	Inf.	Inf.	Inf.	4.87	Inf.	0.13	7.00	Inf.	3.39	1.02	112.1	Inf.	1.45		0.89*	0.59*	0.95*	0.14*	0.71*
Peterborough	0.21	0.19	0.09	0.19	0.47	0.09	Inf.	0.59	0.09	Inf.	0.74	0.14	1.55	0.18	0.06		0.04	0.23	-0.02	-0.09
ParaWirra	0.21	0.80	0.48	0.80	1.12	0.45	Inf.	0.70	0.48	3.79	0.67	0.39	1.18	0.45	0.35	11.02		-0.21	0.12	0.09
Hattah Lakes	0.17	0.15	0.05	0.15	0.39	0.04	Inf.	0.45	0.05	Inf.	0.79	0.08	2.20	0.17	0.03	1.71	Inf.		-0.31	0.29
Bourke	3.94	Inf.	10.93	Inf.	20.26	7.86	Inf.	3.23	10.93	Inf.	3.86	3.53	11.04	Inf.	3.20	Inf.	3.76	Inf.		0.11
Hillston	0.21	0.44	0.28	0.44	0.73	0.27	Inf.	0.67	0.28	4.19	0.65	0.28	1.22	0.28	0.21	Inf.	5.21	1.23	4.23	

Table V-6: Genotypes of grey kangaroos exhibiting introgression of Y-haplotypes. Alleles in bold/shaded indicate introgressed microsatellite alleles, based on the mtDNA haplotype and according to the allelic range of the species outside the region of sympatry (Chapter IV). * denotes intogression of mtDNA

ID	Y-haplotype	mtDNA haplotype	T4-2	IL5	T32-1	G16-1	T46-5	T19-1	T31-1	Pa595	G20-2	G31-1	G26-4	T3-1T
43	N	Wgk1*	122 120	150 150	163 163	160 179	163 167	127 123	130 120	232 252	**145** 157	130 136	355 275	**[illegible]**
129	A	EGKy46	120 120	140 160	170 168	172 180	167 163	151 151	128 140	236 232	151 147	136 136	347 355	181 251
150	A	EGKy6	122 122	146 146	160 178	160 170	159 167	143 155	128 126	236 236	151 155	128 **134**	271 263	181 189
161	A	EGKy7	120 120	140 160	176 176	164 180	167 **151**	143 143	126 128	232 232	147 153	**134** 132	343 367	253 157
180	A	EGKy9	122 120	146 156	160 172	164 184	167 167	**163** 167	126 124	236 244	151 149	130 122	339 271	185 281

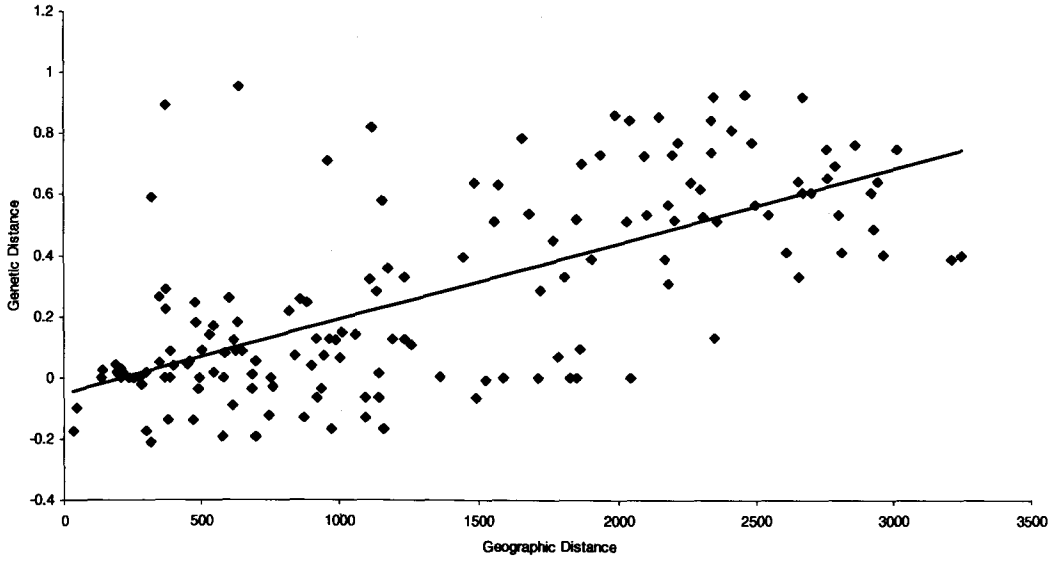

Figure V-3: Plot of genetic distance (R_{ST}) and geographic distance (km) between mainland *Macropus fuliginosus* populations ($p<0.001$; $R^2=0.46$). The line indicating the least squares regression estimate.

Discussion

Overall the amplification of Y-linked microsatellite loci isolated from *M. eugenii* was successful in *M. fuliginosus*, displaying comparable levels of diversity (Macdonald *et al.*, 2006). However, as can occur in cross-species amplification, inconsistent banding patterns were evident in one of the 5 loci. In general it appears that like autosomal microsatellite markers (Zenger *et al.*, 2003b), Y-linked microsatellite markers isolated from *M. eugenii*, will prove useful in a range of other macropod species.

Western Grey Kangaroo

Relatively little microsatellite diversity was apparent on the Y-chromosome in *M. fuliginosus* with only 0.07 haplotypes found per sample analysed. This low diversity may be associated with the female biased sex ratio (3:1, Norbury *et al.*, 1988) and polygynous mating system (Strahan, 1995), where only a few dominant males contribute to subsequent generations in *M. fuliginosus*. Furthermore, the low levels of Y-chromosome diversity are in contrast to the high levels of diversity apparent in the mtDNA control region (Chapter III), which may also be attributed to polygyny and the unequal contribution of the sexes. A similar reduction in diversity on the Y-chromosome has been reported in hamadryas baboons (Lawson Handley *et al.*, 2006) and some human populations (Kayser *et al.*, 2003). However, examination of more Y-linked loci and a larger sample size may reveal a greater number of haplotypes within the sampled populations.

Males are assumed to be the dispersing sex in macropods (Johnson, 1989). However, ecological studies in *M. fuliginosus* indicate males are largely sedentary, with 90% of individuals moving less than 6 km (Priddel *et al.*, 1988) and several juvenile males observed to remain within the natal area (Arnold *et al.*, 1992). In contrast to the ecological data, Y-chromosome analysis reveals high levels of migration, with few sites displaying significant differences. This disparity is not surprising since previous studies were typically restricted to adult individuals in which natal dispersal has already occurred. A single sub-adult male for instance travelled approximately 85 km for its point of capture (Priddel *et al.*, 1988), the type

of movement which may be associated with natal dispersal. This movement placed the individual outside the study area; hence large movements are rarely detected using most previously employed techniques. The Y-chromosome data is consistent with the levels of male migration implied by the comparison of maternally and bi-parentally inherited markers (Chapters II & III). However, the small sample sizes reduces the power to detect migration and further sampling would be required to ascertain if male dispersal is as high as this data indicates.

Three boundaries were previously identified across the mainland range (Chapter II). However, only one of these, the Lake Torrens/Flinders Ranges was evident in the partitioning of Y-haplotypes. This region was also the only boundary which displayed little evidence of admixture (Chapter II). Hence the high levels and relatively widespread admixture present at other mainland boundaries may be largely attributed to high level of male dispersal across the apparent boundaries. The impact of the Lake Torrens/Flinders Ranges region on the population structure of *M. fuliginosus* is not surprising since this region lacks the preferred habitat of *M. fuliginosus* and a corresponding reduction in population densities is apparent (Cairns *et al.*, 2000). The impact of areas of high topographic complexity on large macropods has previously been noted in *M. rufus* (Clegg *et al.*, 1998). Furthermore, this region also appears to present a substantial barrier to dispersal in other species, resulting in population subdivisions in species of birds (Degnan and Moritz, 1992) and reptiles (Fairbairn *et al.*, 1998).

The origin of the Kangaroo Island population has been uncertain, with Kangaroo Island animals appearing similar to eastern mainland *M. fuliginosus* for some traits but also showing similarity with Western Australian *M. fuliginosus* for others (Clark and Poole, 1973; Mead, 1985a; Poole and Catling, 1974). One unique Y-haplotype (D) was identified on Kangaroo Island in approximately equal proportions to the most common Western Australian haplotype (A). Thus, Kangaroo Island appears more similar to Western Australian *M. fuliginosus* supporting the hypothesis of Clarke and Poole (1973) that Kangaroo Island and Western Australia share a common ancestry. This however is discordant with evidence from mtDNA data, which indicates the maternal lineage is derived from mainland individuals east of the Lake Torrens/ Flinders Ranges area. Both Kangaroo Island and the eastern

mainland populations were sourced from south-western Australia, where the species originated and haplotype A appears to represent an ancestral haplotype. Therefore, isolation and drift appear to have resulted in a unique variation in several traits, some of which appear more similar to distant populations. Possible gene flow between Coffin Bay Peninsula and Kangaroo Island prior to its isolation, resulting in differing origins for male and female lineages is another potential explanation for observed results. However, haplotype A is not absent from the eastern populations, although its frequency is reduced. Further examination of variation on the Y-chromosome will be required to more conclusively elucidate the origins of the paternal lineage of *M. fuliginosus* on Kangaroo Island.

Hybridisation

Hybridisation between the two species of grey kangaroos has previously been identified in the region of sympatry (Chapter IV). However, the levels of hybridisation are low and more indicative of occasional breakdowns in species boundaries, rather than a hybrid zone. The results presented here for the Y-chromosome are consistent with this low level of hybridisation, with only four individuals displaying introgressed Y-haplotypes.

In captivity the breakdown in species recognition was typically observed to result from male *M. fuliginosus* hybridising with *M. giganteus* females, the reciprocal cross was never observed (Kirsch and Poole, 1972; Poole and Catling, 1974). Male *M. giganteus* were observed to be less likely to recognise female *M. fuliginosus* in oestrus and were also less likely to breed with hybrid females, although this cross was successful (Kirsch and Poole, 1972; Poole and Catling, 1974). Although introgression in the region of sympatry was evident in both directions, a slightly large number of hybrids identified were associated with the *M. giganteus* maternal lineage (Chapter IV). Therefore, evidence of introgression of *M. fuliginosus* Y-chromosome haplotypes into *M. giganteus* populations is not surprising and reflects the predominately unidirectional nature of hybridisation in grey kangaroos.

The occurrence of Y-chromosome introgression in grey kangaroo species is, however unusual, as F1 males were presumed to be infertile. Examination of 3

captive bred F1 males revealed that although the tubules were open in 2 of the 3 individuals only primary spermatogenesis was apparent (Poole and Catling, 1974). The single F3 backcrossed male (female mated back to *M. fuliginosus* male) however, was fertile and development appeared similar to pure bred animals (Poole and Catling, 1974). The restoration of male fertility with backcrossing is not uncommon and has previously been observed in other species (e.g. Zong and Fan, 1989) and presents a potential means of Y-chromosome introgression. The limited number of hybrid individuals examined and the variation present within them, does not exclude the possibility of the occurrence of fertile male F1 hybrids which could result in the observed introgression. However, the restored fertility of F3 males coupled with the approximately 60,000 years of secondary contact (Chapter III), may have allowed combinations of individuals, exhibiting various degrees of backcrossing to breed resulting in the rare instances of introgression of Y-chromosome which were apparent in this study.

Potentially introgressed autosomal microsatellite alleles were apparent in three of the four individuals displaying Y-haplotype introgression. Although these alleles were not found in the 20 control *M. giganteus* individuals (Chapter IV), they still fall within the allele ranges of both species and alone are insufficient to indicate a potential hybrid ancestry. This highlights the need for examination of a large number of loci, greater than 48 according to Vaha and Primmer (2006) to detect the low levels of introgression which are evident in grey kangaroos. Therefore further investigation of more loci may reveal more hybrid individuals. The results of this study also confirmed the classification of kangaroo ID 43 as a *M. giganteus* backcross, with a *M. giganteus* Y-haplotype and authenticated it as the sole case of mtDNA introgression identified thus far.

Hybridisation among grey kangaroos appears to result from rare and potentially individual specific breakdowns in the species boundaries. No clear patterns in the direction of hybridisation are evident within the region of sympatry and introgression in Y-haplotypes (this study) and both microsatellite and mtDNA (Chapter IV) are apparent. Therefore complex interactions between within grey kangaroo species appear to operate within the region of sympatry, potentially

resulting from a long period of secondary contact and near complete pre-zygotic barriers to reproduction.

Conclusion

The successful amplification of these loci indicates the potential utilisation of these markers in a variety of macropods. Examination of the Y-microsatellite loci in *M. fuliginosus* has revealed low levels of diversity reflective of skewed sex ratios and polygyny. High male dispersal is evident in *M. fuliginosus* and the admixture previously identified boundaries may be attributed largely to male dispersal across these barriers. The Lake Torrens/ Flinders Ranges region however, appears to restrict the free movement of male *M. fuliginosus* resulting in the low levels of admixture observed. Finally introgression of the *M. fuliginosus* Y-chromosome into *M. giganteus* populations was evident, further revealing rare hybridisation events occurring within the region of sympatry. However no clear patterns of hybridisation in grey kangaroos was evident.

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CHAPTER VI:

General Conclusions

Genetic markers have proven useful in exploring many facets of species biology, including population history and structure, as well as dispersal. However, relatively few studies on macropods have thoroughly incorporated molecular techniques. In this study both autosomal and Y-chromosome microsatellite markers, as well as the mitochondrial DNA (mtDNA) control region were used to examine various aspects of the biology of the western grey kangaroo, *Macropus fuliginosus*, one of the largest and most widespread species of macropod. The results of this study produced some of the most detailed genetic evidence for the contemporary structure, phylogeography and population history of any species of kangaroo, with several aspects investigated for the first time in marsupials.

Despite its current widespread distribution *M. fuliginosus* evolved separately, on the western side of the continent, from its sister species the eastern grey kangaroo, *M. giganteus* (Figure VI-1a). According to the degree of differentiation in their mtDNA control region sequences (Chapter III), the two species diverged almost one million years ago, early in the Pleistocene. The climatic fluctuations of the Pleistocene subsequently profoundly influenced genetic structure within *M. fuliginosus*. Approximately 150,000 years ago (Chapter III), during the penultimate glacial (arid) phase (250-120,000 years ago, Nanson *et al.*, 1992) a division between the northern and southern regions of the distribution appears to have resulted in the two divergent lineages apparent in phylogenetic analyses of mtDNA control region sequences (Figure VI-1b; Chapter III). The increased water and habitat availability in the subsequent interglacial phase (Bowler, 1976; Nanson *et al.*, 1992) appears to have then initiated an eastward range expansion in *M. fuliginosus*, with the invasion of the Nullarbor Plain, approximately 80,000 years ago (Figure VI-1c; Chapter III). The far eastern regions of the range were reached some 20,000 years later and the majority of the current distribution appeared to be colonised prior to the last glacial phase (Figure VI-1d; Chapter III). This eastward expansion was the result of a relatively small invading population according to mismatch analysis (Chapter III) and is consistent with the microsatellite evidence indicating sequential founding events, which resulted in a progressive reduction in the degree of genetic diversity at autosomal microsatellite loci from west to east (Chapter II). Following the initiation of the eastward expansion, the obstruction to gene flow between the northern and southern regions of the western range was reduced resulting in the

contemporary admixture which is apparent at both microsatellite (Chapter II) and mtDNA haplotypes (Figure VI-1d; Chapter III).

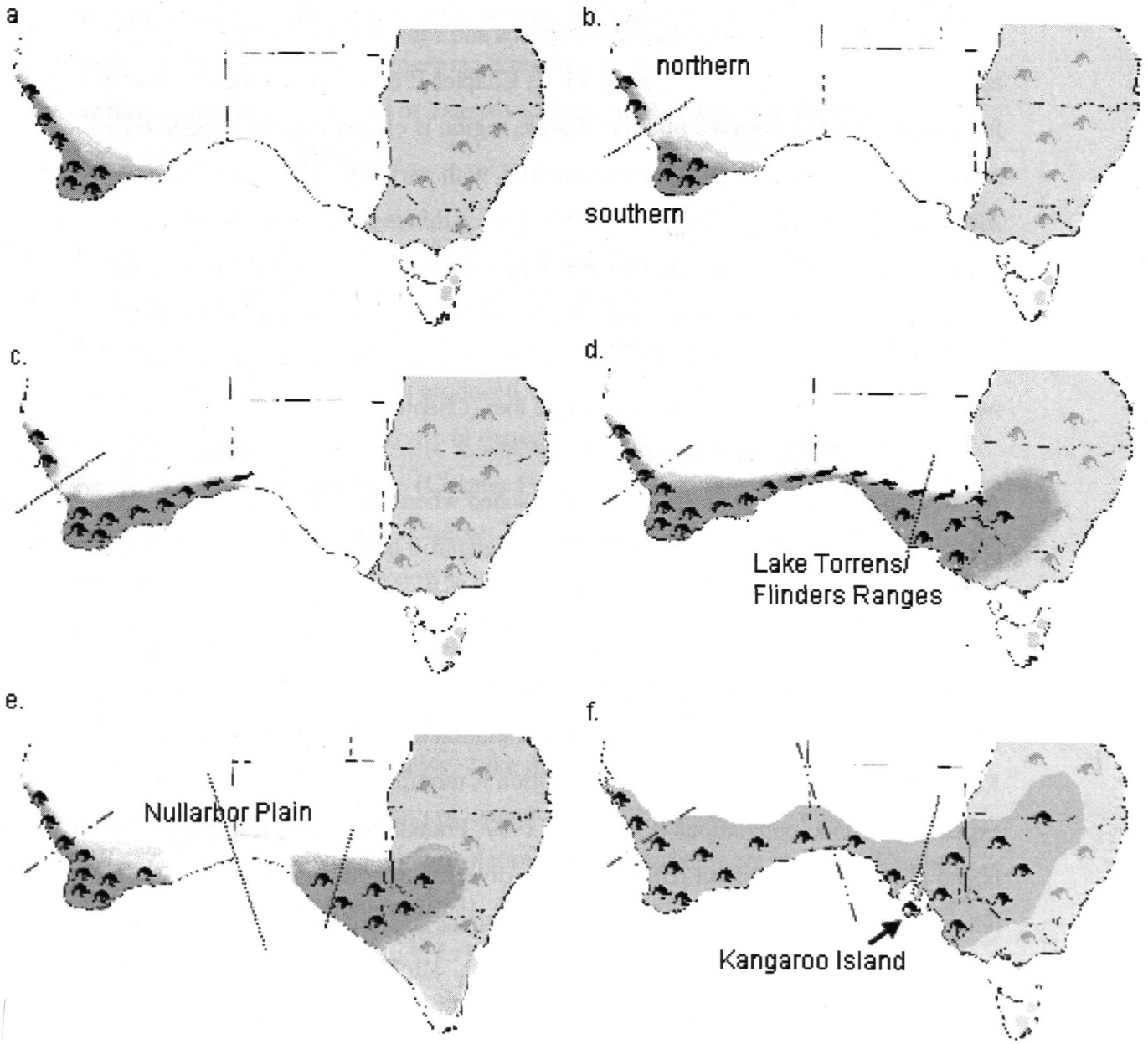

Figure VI-1: The Pleistocene distributional changes and filters identified in *Macropus fuliginosus* (grey with black kangaroo symbols). The current distribution of its sister species *Macropus giganteus* is shown in light grey. a. ~1 million years ago; b. ~150,000 years ago; c. ~80,000 years ago; d. ~60,000 years ago; e. ~18,000 years ago; f. ~9,500 years ago to present day. ····· indicates the presence of active filter, while —··—·· indicates the filter no longer appears to significantly restrict dispersal.

The most recent of the last glacial phases began approximately 25,000 years ago, peaking around 18,000 years ago (Bowler, 1976; Bowler, 1982). During this time the Nullarbor Plain reached its driest point and was likely to present a significant barrier to gene flow resulting in the two genetic units apparent in the autosomal microsatellite data (Figure VI-1e; Chapter II). Subsequently, this filtering effect has been reduced with the end of the glacial phases and substantial admixture is apparent at microsatellite loci (Figure VI-1f; Chapter II & V). In contrast the barrier formed by the Lake Torrens/ Flinders Ranges region is clearly evident in both Y-chromosome and autosomal microsatellite loci, with very little evidence of recent admixture (Figure VI-1f; Chapters II & V). Hence this region appears to present an extensive and contemporary filtering effect on gene flow, restricting the movement of male *M. fuliginosus*, the dispersing sex according to comparisons of migration rate derived from mtDNA and autosomal microsatellites (Chapter III) as well as from direct examination of Y-microsatellite loci (Chapter V).

A situation similar to the present day was reached when sea levels rose, approximately 13,000 years ago and isolated Kangaroo Island and its biota from the mainland around 9,500 years ago (Figure VI-1f, Thom and Chappel, 1975). Kangaroo Island appears to have been colonised during the eastward expansion (Chapter III). The maternal (mtDNA, Chapter III) and paternal (Y-chromosome, Chapter V) lineages indicated contrasting population histories for the sexes on Kangaroo Island, although further examination is required to confirm this. As is expected of island populations (Frankham, 1997; Frankham, 1998), Kangaroo Island *M. fuliginosus* exhibit reduced genetic diversity at both autosomal loci (Chapter II) and the mtDNA control region (Chapter III). Furthermore, although no ancient genetic bottleneck is associated with colonisation of the island (Chapter III), a recent genetic bottleneck (Chapter II) appears to have resulted from extensive hunting of the kangaroos during the early 1800's shortly following their discovery (Clumpston, 1986; Flinders, 1814).

In contrast to previous ecological research which indicated that *M. fuliginosus* was largely sedentary (Arnold *et al.*, 1992; Priddel *et al.*, 1988), high levels of dispersal were apparent in the genetic data. This is not surprising as ecological studies were largely restricted to adult individuals in which natal dispersal had already occurred.

A relationship between geographic and genetic distance in both autosomal (Chapter II) and mtDNA (Chapter III) only exists in the western regions of the range as a result of the recent (Pleistocene; Chapter III) expansion into the eastern areas or differences associated with environmental heterogeneity. The influence of increased topographic complexity, for instance, is apparent in some populations of *M. fuliginosus* (Chapter II) as well as in other macropod species (Clegg *et al.*, 1998). In *M. fuliginosus*, however male patterns of dispersal only appear reduced by large distances (>1000km) or extensive filters such as the Lake Torrens/ Flinders Ranges barrier (Chapter V).

Despite difficulties associated with hybridisation between grey kangaroos, *M. fuliginosus* and *M. giganteus* in captivity, rare and potentially ancient hybridisation events were evident in the region of sympatry. Hybridisation in captivity appeared unidirectional and the F1 males produced were infertile (Kirsch and Poole, 1972). However, the increased sensitivity of genetic techniques revealed evidence of introgression in both directions (Chapter IV) and four individuals displayed introgression of Y-haplotypes (Chapter V). These levels of introgression are indicative of occasional breakdowns in reproductive isolation, rather than a discreet contact zone.

The information revealed by genetic analyses in *M. fuliginosus* also has implications for the management of this species. Like many species of large macropod, *M. fuliginosus* presents a dichotomy of management options. In many areas *M. fuliginosus* is overabundant and population control measures are required (Coulson, 2001; Shepherd and Caughley, 1987). High densities of large macropods may not only present a significant economic loss but may also hamper conservation efforts in other species (Barnes and Hill, 1992; Coulson, 1998). Understanding the dispersal abilities and demic structure of *M. fuliginosus* will enhance the effectiveness of control measures (Viggers and Hearn, 2005). Furthermore, *M. fuliginosus* is commercially harvested throughout most of its range for its meat and skin. Appropriate units and scales of management across the range were identified using genetic techniques and current management practises, for the most part, appear relevant. However, several separate management units exist across the range and these should be incorporated into future management schemes (Chapter II).

Furthermore, current taxonomic classification of the Kangaroo Island subspecies requires revision as this population does not appear to possess a major component of the genetic diversity in *M. fuliginosus* (Chapters II & III).

Genetic markers are capable of elucidating patterns and population histories, which are not apparent using other methodologies. Furthermore, they provide a means of quantifying the extent of dispersal and the filtering influence of features in the landscape, both contemporary and historic. Although *M. fuliginosus* is an abundant and widespread species of macropod, the genetic data obtained in this study has revealed new insights into dispersal, contemporary structure and the influence of Pleistocene climatic changes as well as evidence of hybridisation with *M. giganteus*. Although similar studies have been conducted in relatively few macropod species, it is obvious that molecular techniques are capable of providing new information on macropods, which is relevant to their conservation and management.

Future Research

This genetic study of *M. fuliginosus* has addressed many of the current gaps in our understanding and yielded valuable insights which will aid in the future management of this species. However, several questions remain unexplored and many more have been raised by the results of this study.

Some minor subdivisions were apparent within populations of *M. fuliginosus* and may reflect the presence of multiple social groups within sites (Chapter II). Although females appear philopatric and may form the basis of social groups, these associations appear to frequently alter in *M. fuliginosus* and may not be associated with sociality (Arnold *et al.*, 1990). Group structuring and its association with relatedness is yet to be examined in *M. fuliginosus* using genetic techniques.

The impact of environmental heterogeneity and resource availability as well as the influence of landscape features, such as creek lines in arid areas, on dispersal requires further investigation in *M. fuliginosus*. More extensive sampling to account for potential dispersal routes and stratification into differing environmental or climatic regions will aid in further elucidating these influences. Similarly, further

sampling of the region in the Nullarbor Plain as well as in the Lake Torrens/ Flinders Ranges region is required in order to establish the full extent to which these landscape features act as filters/barriers to gene flow and dispersal, both in an historical and contemporary context. Additionally, further sampling in the southern regions of the eastern range, particularly on Fleurieu and Yorke Peninsulas will aid in elucidating the origins of the paternal lineage on Kangaroo Island.

M. fuliginosus is the first macropod species in which Y-chromosome markers have been used to assess variation across populations. The low levels of variation detected, however, mean that further sampling of male individuals, as well as examination of additional Y-markers, will be required to more accurately assess the levels of variation and population histories, such as Kangaroo Island. Furthermore, the potential association between low Y-chromosome diversity and the mating system also highlights the need to investigate the mating system of *M. fuliginosus*, knowledge of which is currently restricted to observational and captive studies.

The increased sensitivity of genetic techniques has revealed previously undetectable levels of introgression between *M. fuliginosus* and *M. giganteus*. Although no patterns of introgression were evident in this study, further examination of hybridisation across the region of sympatry to account for changes in the respective densities of the two species may elucidate the existence of any potential patterns. Furthermore, sampling of pouch young, particularly those produced following drought and/or reduced densities of the two species may reveal some of the potential reasons for the occurrence of hybridisation in grey kangaroos.

In addition to highlighting several aspects of the biology of *M. fuliginosus* which require further investigation, this study has also opened several new lines of enquiry in other species. For instance, several other species of macropod are capable of hybridising in captivity and occur sympatrically in some part of their ranges (Close and Lowry, 1990). All are yet to be examined for evidence of introgression. Similarly, the influence of Pleistocene climate fluctuations on gene flow and range changes across southern Australia has only been examined in relatively few species. Furthermore, the cause of the barrier/filter which resulted in the temporary historic division between northern and southern *M. fuliginosus* mtDNA lineages in the

western region of the range remains unknown. Further investigation is required to see if a similar phylogenetic break occurs in other sympatric species. Similarly the impact of the remaining mainland barriers/filters identified in *M. fuliginosus* on the genetic structure of other species which share this distribution also requires additional examination.

The results of this study revealed several aspects of the genetic structure and population history of *M. fuliginosus*, knowledge of which was previously restricted to ecological and captive based studies. For the first time in a macropod species variation on the Y-chromosome was assessed across populations. Furthermore, this research revealed the history of *M. fuliginosus*, including the influence of the Pleistocene climate fluctuations as well as contemporary barriers/filters within the mainland range. Since *M. fuliginosus* is the first widespread southern Australian species to be examined in detail, these features may also prove capable of similarly influencing the structure of other extant Australian fauna. Finally all this information has practical implications for the management of *M. fuliginosus*, which is harvested throughout most of its range. Relatively few macropod species have been examined to this degree, yet this study reveals the capacity of molecular techniques to provide new and valuable information and the importance of such investigations to macropod research.

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