CHAPTER 1

LITERATURE REVIEW

1.1 THE MAMMALS

Mammals are distinguished from other vertebrates by their jaw structure, the possession of hair and of the eponymous mammary gland. The class Mammalia consists of three subclasses: prototheria, metatheria and eutheria. These three groups are distinguished largely by their modes of reproduction. The prototheria or monotremes are egg-laying mammals. The metatheria or marsupials give birth to immature young which continue development whilst attached to an external teat which in many species is contained in a pouch. Eutherians give birth to more mature young which are initially nurtured during a prolonged period of interuterine life. The prototheria diverged from the therian lineage between 150 and 200 million years ago (Kemp 1982). Eutherians and metatherians diverged between 80 and 170 million years ago (Hope et al., 1990; Novacek 1992). It is common to regard metatherians and prototherians as relic forms of mammals which have only managed to survive in this "primitive" state because of lack of competition from eutherians (Tyndale-Biscoe, 1973; Kirsch and Calaby 1977). The fact that both marsupials and eutherians co-exist in the Americas argues against this idea. Contemporary Australian zoologists point out that marsupial and monotreme species have evolved highly specialised characteristics for their particular environments as is reflected in the ecological diversity within the two groups. For example, marsupials include such distinct species as the herbivorous, bipedal "giant" red kangaroo (*Macropus rufus*) weighing up to 80kg and the small, fossorial, sightless marsupial mole (*Notoryctidae typholops*). Perhaps as a consequence of this misconception mammalian biology is frequently discussed in terms of eutherians only, with marsupials and monotremes being considered as a minor variation on the main theme.

1.2 THE MARSUPIALS

Extant marsupials are confined to the Australian biogeographic region (Australia, New Guinea and parts of Indonesia) and the Americas (Kirsch and Calaby 1977). Marsupials are frequently divided into three orders: the Paucituberculata (which are restricted to the Americas), the Diprotodonta (containing possums and macropodids which are restricted to the Australian region) and the Polyprotodonta (which includes marsupials from both regions, specifically containing carnivorous dasyurids and omnivorous bandicoots from the Australian region). There are about 250 species of which two-thirds are found in the Australian biogeographic region where they are the dominant group of mammals. There is general agreement on the classification of marsupials to the family level. However higher order classification has proven problematic and has been the subject of great debate (Kirsch and Calaby 1977).

1.2.1 Marsupial Genetics

The DNA content of marsupials and eutherians is similar (Hayman and Martin 1974) but marsupials tend to have fewer and therefore larger chromosomes (Hayman 1990). The marsupial range is 2n=10 to 32 with two strong modes of 2n=14 and 16 (Hayman 1990) compared to the eutherian range of 2n=6 to 84 (reviewed in White 1973 p.391). Many marsupial karyotypes appear to be conserved over wide evolutionary distances (Hayman et al., 1987; Hayman 1990), suggesting that the rate of karyotypic evolution in marsupials may be slower than in some eutherian lineages. There is evidence that a 2n=14 karyotype represents the plesiomorphic (ancestral) state for marsupials (Rofe and Hayman 1985). This high degree of conservation makes it potentially possible to generalise about marsupial genome structure and consequently the marsupial genetic map.

Comparative Genetics

The number of species for which genetic maps are being developed has increased substantially over the last decade (O'Brien 1993). Specific research questions such as location of disease loci in humans and identification of genes associated with production traits in agriculturally important animals have aided and added to the development of genetic maps. There are however, far broader reasons for the generation of such maps. Firstly, to increase understanding of detailed species-specific genome structure and secondly, and more generally, to gain insight into mammalian genome evolution. To date, the majority of available maps are for eutherian species - the most comprehensive being for humans (HGM 11 1991) and mice (HGM 11 1991; Copeland and Jenkins 1991; Avner et al., 1988). Comparisons between different species maps has led to the identification of many conserved groups of linked genes across varying evolutionary distances. As marsupials and eutherians diverged between 80-170 million years ago (Hope et al., 1990; Novacek 1992), comparisons between marsupial and eutherian gene maps will add a new dimension to the understanding of mammalian genome evolution. Comparing such divergent genomes may help elucidate the evolution of genetic linkage groups and to identify ancient therian chromosome structure and organisation. More specifically, comparisons between marsupials and eutherians may provide further understanding of gene regulation and expression in mammals.

In comparison to the wealth of data available for eutherian species, few loci have been mapped in marsupials (reviewed in Graves et al., 1993). There have been gene assignments made in only 13 species which are all derived from just four families: the Macropodidae, the Phalangeridae, the Dasyuridae or the Didelphidae. So it follows that there are twelve families and about 237 species of marsupials for which no information is available. In addition, there is very limited data for some studied species. For example, in *Didelphis virginiana* (Didelphidea) only three gene assignments have been made - all to the X chromosome (Kaslow and Migeon, 1987; Samollow et al., 1987). The species for which most information is available is the

tammar wallaby (*Macropus eugenii*: Macropodidae) where 37 genes have been assigned either to chromosomes, genetic linkage groups, or both.

A number of techniques have been employed in marsupial gene mapping with varying degrees of success. Approximately fifty genes have been assigned to the X and autosomes by somatic cell genetics (reviewed in Graves et al., 1993). However marsupial/eutherian hybrids cells are unstable and recovery rates are low. The few hybrids recovered often do not contain recognisable marsupial chromosomes due to loss, fragmentation and rearrangement. This, together with difficulties in identifying expressed gene markers of both marsupial and eutherian origin that exhibit distinguishable genetic variation, has restricted the use of somatic cell hybrids in marsupial gene mapping.

Over forty gene assignments have been made to marsupial chromosomes by *in situ* hybridisation (reviewed in Graves et al., 1993). The greatest difficulty with this approach is the lack of suitable gene probes. Very few marsupial sequences have been cloned, although the number has increased substantially over the last five years (Collet et al., 1989; Collet et al., 1990; Cooper and Hope 1993; Fitzgerald et al., 1993; Kusewitt et al., 1994; Richardson et al., 1994; Collet and Joseph 1994.). The problem of obtaining suitable probes has been overcome to some extent by the use of heterologous probes. Cloned sequences from other organisms (generally eutherian) if sufficiently conserved, can be localised to marsupial chromosomes. When the time of divergence of marsupials and eutherians is considered it is clear that many genes will not share the degree of homology necessary for this approach to be successful.

As opposed to physical mapping approaches, several groups have used classical gene mapping to assign genes to linkage groups through recombination. Previous to this work only five linkage groups had been identified. These are ADA-GPI-PI- and PGD-SOD-TRF in the Australian species *Sminthopsis crassicaudata*, (the fat-tailed dunnart) (Bennett et al., 1986); AKA1-PI and C6-C7-GPT in the distantly related American marsupial, *Monodelphis domestica* (the gray short-tailed opossum) (van Oorschot et al., 1992; van Oorschot pers. comm.); and GPI-PI in

tammars (van Oorschot and Cooper 1990). This paucity of information results largely from the difficulty of identifying a marsupial species which breeds well in captivity, exhibits sufficient and detectable genetic variation, and has a short generation time and/or produces enough offspring for linkage analysis.

Conservation Genetics

The distinctiveness of the Australian fauna results from its long isolation from the faunas of other land masses since separation from the supercontinent of Gondwana early in the Tertiary, some 45 million years ago. Sadly, Australia's distinctiveness does not end here. Australia has the worst extinction rate for mammals of any continent or country and accounts for about half the world's mammals that have become extinct in modern times (reviewed in Kennedy 1992).

There are ten Australian marsupial species known to be extinct but this is probably an underestimate as others might have reached extinction before they were recognised and documented by naturalists. Australia now has 140 species of marsupials of which 27 are currently listed as vulnerable or in danger of extinction (Burbidge and N. McKenzie 1989; CONCOM 1991). The urgent need for action to conserve Australia's native fauna is finally receiving a great deal of interest and discussion (Kennedy 1992). There have been considerable advances in marsupial conservation in recent years. Knowledge of species ecology is growing rapidly, there is a developing science of species management and there is a heightened interest in conservation in the country. Management plans for endangered species such as captive breeding and reintroduction programs are currently being debated in the literature. One of the underlying considerations of these programs is the assessment and maintenance of genetic variability and the population relationships within the species under threat.

1.3 THE MACROPODIDAE

The superfamily Macropodidea contain 57 extant species distributed throughout Australia, New Guinea and Irian Jaya, and has been described as the most successful group in the Australian fauna (Tyndale-Biscoe 1973). About 80% of macropods have been studied cytologically (Hayman 1990). They are the most chromosomally diverse group of marsupials with diploid numbers ranging from 2n=32 (*Aepyprymnus rufescens*, the ~~rufus~~ rufous bettong) to 2n=10,11 (*Wallabia bicolor*, the swamp wallaby) (reviewed in Kirsch and Calaby 1977). Two families are recognised within the superfamily, the Potoroidae or small rat kangaroos which are confined to Australia, and the Macropodidae which are found throughout the Australian biogeographic region. *Macropus*, the largest genus within the Macropodidae includes the typical kangaroos and large wallabies and two small wallabies. Species are restricted to Australia with the exception of *M. agilis* (the agile wallaby) which is distributed throughout the Australian biogeographic region. Of the 14 recent species, two are considered potentially vulnerable or vulnerable to extinction (the tammar wallaby and *M. irma* - the Western brush wallaby), one was thought to be extinct for many years (the parma wallaby) and one is now extinct (*M. greyi* - the toolache wallaby) the last specimen of which died in captivity in 1939 (Robinson and Young 1983). All members of the genus *Macropus* have a diploid number of 2n=16 except the *M. rufus* (the red kangaroo) which is 2n=20. and the black wallaroo (*M. bernardus*) which is 2n=18 (Sharman, unpublished data)

1.3.1 The Study Species

Macropus eugenii (the tammar wallaby)

In 1629 Francisco Pelsaert, captain of the Dutch East India Company ship "Batavia" was shipwrecked on the Abrolhos Island group off the Western Australia coast. It was here that he discovered the tammar wallaby. His description is the first known of an Australian macropodid. Whilst common on some islands, tammars are considered vulnerable to extinction (Kennedy 1992). Their former range included semi-arid areas of the south west of Western Australia and South Australia, five

Western Australian islands and four South Australian islands. They remain on all five Western Australian islands (East and West Wallabi (or Houtman and Abrolhos respectively), Garden, Middle and North Twin Peaks) and are abundant on Kangaroo Island in South Australia. There are remnant populations in south west Western Australia, but tammars are probably extinct on mainland South Australia and three South Australian islands (Flinders, St. Peter and Thistle). Tammars have been introduced to Granite, Greenly and Boston islands in South Australia and to Kawau Island and a large area around Rotorua in New Zealand.

Tammars are small wallabies, weighing between 5 and 8 kg. It is possible to hold relatively large numbers in captivity where they have been shown to breed well. The biology of the Kangaroo Island tammar wallaby has been studied intensively for over a decade (Hinds et al., 1990). Some of the main advances have been in the understanding of its reproductive biology. Females reach sexual maturity at about 12 months and males at about 18 months. The gestation period is approximately 28 days. There is a defined breeding period (Berger 1966) which is controlled by day length (Tyndale-Biscoe et al., 1986). Most females carry a dormant blastocyst throughout the year which becomes reactivated after the summer solstice as daylength begins to shorten. Immediately after giving birth the female mates. The embryo develops to about the 80-160 cell stage after which it enters diapause until the next summer solstice (Tyndale-Biscoe 1979). Reactivation of the embryo is also under the control of lactation - this is known as lactational quiescence. Whilst the pouch young is suckling, the embryo remains in diapause. However loss or removal of the pouch young induces reactivation of the dormant blastocyst (Merchant 1979; Hinds and Tyndale-Biscoe 1982). The success of reactivation by removal of pouch young diminishes after the winter solstice. None the less, it is possible to induce reactivation through this period by one of three experimental procedures. The first is the administration of the drug bromocriptine, an inhibitor of prolactin production. A single intramuscular injection of 5 mg/kg of bromocriptine leads to the reactivation of the dormant blastocyst. This approach is effective between April and September

(Tyndale-Biscoe and Hinds 1984; Tyndale-Biscoe et al., 1986). Secondly, reactivation will occur if tammars are held on a photoperiod regime of equinoctial daylength (12L:12D) (Sadleir and Tyndale-Biscoe 1977). Thirdly, administration to females held on 15L:9D of 440ng/kg of melatonin 2.5 hours before lights off for 3-5 days will induce reactivation of the blastocyst (Hinds and den Ottolander 1983; Tyndale-Biscoe et al., 1986). Hence, whilst tammars usually only have one offspring per year, it is possible through removal of pouch young or experimental manipulation to induce a female to produce (theoretically) up to twelve offspring per year. This maximum is unlikely to be reached (see Chapter 3 of this thesis).

Macropus parma (the parma wallaby)

Parma wallabies have no defined breeding season, but significantly more births occur in January-June than July-December (Maynes 1977). The gestation period is about 35 days and pouch life is approximately 30 weeks (Maynes 1983). Females are sexually mature by 12 months of age (Maynes 1977). Ovulation, mating and a subsequent birth can, but does not always, occur before a previous pouch young is weaned (Maynes 1977). Males are sexually mature by 98 weeks (Maynes 1976a).

Macropus dorsalis (the black-striped wallaby)

There is a great paucity of information regarding the biology of the black-striped wallaby. Females are sexually mature by about 14 months and males by 20 months. Females come into oestrus shortly after giving birth. Gestation is 33-35 days and pouch life is 210 days (Kirkpatrick 1983). An intensive study of aspects of the biology of black-striped wallabies is currently being undertaken by Mr. Peter Johnson, Queensland National Parks and Wildlife Service, Townsville, but to date data from these studies are not available (P. Johnson, pers comm).

CHAPTER 2

AIMS

This research was begun with two central aims in mind. The first was to develop an efficient system for mapping a marsupial genome through recombination. The second was to generate data which would allow conclusions to be drawn about the taxonomic relationships, genetic variability and conservation status of different populations of tammars, and of two other species, parma and black-striped wallabies. The two aims were closely interconnected; it was envisaged that both the breeding and genetic typing data to be collected would serve both purposes.

Two different systems were assessed for their potential usefulness for the mapping work. One was an interspecies cross between parma and black-striped wallabies, and the other a cross between KI and GI tammars. The interspecies cross was wider and likely to generate more genetic markers through fixed differences while hybridisation between what were regarded as two races of tammar was more likely to be successful. At the outset it was felt desirable not to be dependent upon one system. Breeding and mapping of tammars are described in Chapters 3 and 5. Breeding of parma and black-striped wallabies is described in Chapter 7.

The particular conservation and taxonomic questions were as follows.

1. What is the taxonomic status of KI and GI tammars. In particular, is it possible to answer the question: Are KI and GI tammars distantly related races or closely related species? (Chapter 4)

2. What effect does small population size have upon genetic variability on island population of macropodids? (Chapter 4)

3. To what extent is gene flow possible between parma and blackstripes? (Chapter 7)

As this work progressed it became clear that information would be obtained regarding the level of genetic polymorphism at MHC class II loci at the DNA level in

tammars. In the past, indirect results had suggested that genetic variablity at these loci was reduced in marsupials. It became clear that this work would provide an opportunity to examine this aspect of marsupial biology in a stringent fashion, and so analysis was undertaken. (Chapter 6)

CHAPTER 3

BREEDING WITHIN AND BETWEEN ISLAND POPULATIONS OF TAMMAR WALLABIES

3.1 INTRODUCTION

As discussed in Chapter 2, two of the principle aims of this research were to develop an efficient system for mapping a marsupial genome through recombination and to examine relationships between populations of tammars. A strategy of crossing two subspecies of tammar wallabies, one from Kangaroo Island (KI) in South Australia (SA) and the other from Garden Island (GI) in Western Australia (WA), and then backcrossing F1 hybrids to the parental strains was adopted. The Nei genetic distance between KI and GI tammars is 0.16 based on allozyme and protein data (van Oorschot and Cooper, 1988a; Section 4.3.1.; Cooper and Bell, unpublished data). As the two populations are so genetically distinct it was of pivotal interest for the success of this project to establish whether they hybridise readily. During 1989 F1 hybrids of both sexes had been bred at CSIRO Division of Wildlife and Ecology, Canberra and Macquarie University and had been successfully backcrossed to both parental strains. However, at this time only a few animals had been produced and the efficiency of the system was unknown. As a result of producing progeny for mapping studies a large body of observations were accumulated over 1990, 1991, 1992 and 1993 on reproduction between the subspecies. In addition to KI and GI tammars, wallabies from three other areas of WA are used in breeding programs at Macquarie University. They are from Middle Island (MI), East and West Wallabi islands from the Abrolhos group (AI) and from the Perup (P) region of southwest WA. Few AI and P animals are held at Macquarie and their breeding will not be discussed in detail. This chapter summarises these observations and the information this gives on the efficiency of production of animals for linkage estimation and the degree of reproductive isolation between the WA and SA subspecies.

3.2 MATERIALS AND METHODS

3.2.1 Terminology and Manipulation of the Colony

The standard method of recording parentage of vertebrates, except humans, is mother first and father second. For example, KI×(KI×GI) is a backcross with a KI mother and an F1 father whose mother was KI and father was GI.

A prefix of "T" before an animal number stands for "tammar", likewise "E" stands for *eugenii*. These two prefixes were used at CSIRO and Macquarie University respectively. Some animals originally held at CSIRO have no prefix.

3.2.2 Calculation of the possible number of oestrous cycles

The measure of reproductive efficiency was taken as the number of young born per potential oestrous cycle. This measure will be referred to as "the breeding efficiency (BE)" throughout this thesis. Female tammars usually undergo one oestrous cycle per year. However, this is not always the case for the following naturally occurring or experimental reasons:

i) Removal of Pouch Young.

a) Backcross progeny bred for the mapping project were removed between 10-14 days after birth (when they could be sexed by eye). This practice was continued between the summer and winter solstice (21st December to 21st June). Females included in this part of the project could have had up to five oestrous cycles per year.

b) Pouch young born from other matings were not removed unless too many males were born, in which case some selective removals were carried out. These females would probably have cycled again.

ii) Failure of pouch young survival.

Occasionally pouch young are "lost" from the pouch and the female would probably have an additional cycle.

iii) Failure to postpartum mate or conceive.

It has been reported that 80% of KI tammar females carry a dormant blastocyst throughout the year (Hinds et al., 1990) and give birth in late January. Those that do not conceive at the post partum mating have a non-pregnant cycle in the beginning of the year and give birth in early March. Therefore a percentage of females will have two oestrous cycles during one year.

Thus, due to experimental manipulation such as removal of pouch young, natural biological causes ie. loss of pouch young, or failure to postpartum mate or conceive, females used in this study may have up to five oestrous cycles in one year. Accurate calculation of the number of oestrous cycles of a particular female was not possible. Vaginal swabs of females were not undertaken and so it was not possible to state with certainty whether a female was actually cycling. With this in mind it is clear that values of BE are a lower estimate of the number of young born per oestrous cycle.

3.2.3 Acquisition of Animals

Collections of animals from GI and MI were made in 1987 and from AI and the Perup region in 1989 by W. Poole and N. Simms from CSIRO Division of Wildlife and Ecology, Canberra. These animals were transferred to Macquarie University on various occasions during 1991-1992.

3.2.4 Breeding of Animals

The average size of yards holding tammars at Macquarie University is $385m^2$ with the smallest being $225m^2$ and the largest being $1890m^2$. The numbers of animals held in each yard varied from one buck : three females to one buck : twenty four females. Animals were caught at approximately six week intervals at which time the pouch of each female was inspected. Pouch young which were bred for mapping studies ie. backcrosses were removed 10-14 days after birth (when they could be sexed by eye).

All other pouch young were monitored but were left on the teat except when there was too many males in which case removals were carried out.

3.3 RESULTS

During 1991-1993 breeding programs were begun to produce KI, GI, MI, P, F1 hybrids (KI×GI; GI×KI; KI×MI; KI×P; AI×GI) and backcross tammars. The BE of male and female backcrosses was also investigated. These programs were designed to sustain the colony and to generate backcrosses for linkage analysis. Usually one buck was run in each yard with up to 24 females. Some yards contained only one subspecies of female tammar eg. all KI or MI, others contained a mix of females eg. KI, GI, F1's and/or backcrosses. Details of breeding during this period are summarised in Tables 3.1-3.6.

Table 3.1 lists the number of test-cross progeny born to each F1 hybrid and backcross female during 1992 and 1993. The average number of pouch young born per female was 2.8 in 1992 and 1.8 in 1993, giving a mean of 2.3. Similar information regarding the number of test cross progeny born to KI females (ie. KI×F1 matings) is given in Table 3.3. In 1991, 44 KI females were mated with F1 hybrid bucks. The mean number of births per female was 2.4.

There are significant differences between the BE ratios for 1990, 1991, 1992 and 1993 ($\chi^2_3 = 33.12$, $p < 0.05$; Table 3.2). The data suggest that this heterogeneity is due to a reduced BE ratio in 1990.

The numbers of males and females used for each specific cross in each year are listed in Table 3.3. Comparisons between the BE for each cross indicate significant heterogeneity ($\chi^2_{34} = 175.5$, $p < 0.05$). Breeding data for each type of mating (regardless of year) are presented in Table 3.4. Again there are significant differences in the BE between crosses ($\chi^2_{17} = 101.47$, $p < 0.05$). These data are further summarised in Table 3.5 where all WA subspecies are treated as one group. There are significant differences in the efficiency of pouch young production between each group of matings ($\chi^2_6 = 76.86$, $p < 0.05$). Most data was available for KI×WA

crosses. The BE for this group was 15.7%, which is approximately 1/3 that of the other matings. No heterogeneity is detected when the BE for KI×WA and WA×KI crosses (ie. the production of F1 hybrids) were removed ($\chi^2_4 = 7.44$, $p \gtrsim 0.05$).

In order to discover possible sources of the difference between WA × SA crosses and the other matings, an examination of breeding records by year was made. Table 3.6 details the number of pouch young born to KI females run with GI males over sequential years. The data suggest an increase in the BE after the first year of contact.

3.4 DISCUSSION

The results presented in this chapter are a retrospective analysis of observations made of the success of breeding KI, GI, P, MI, F1 hybrid (KI×GI; GI× KI; KI×MI; KI×P; AI×GI) and backcross tammar wallabies during 1990-1993. Examination of this information sheds light generally on the ability to hybridise WA and SA tammars and specifically on the potential success of using subspecies crosses for gene mapping.

By removal of pouch young 10-14 days after birth, it is possible for a female to produce 4-5 offspring between the summer and winter solstice. Females used to generate test cross progeny were caught every six weeks in anticipation of pouch young removal. In practice each female produced a mean of 2.35 offspring per year, approximately half of the theoretical maximum. The actual numbers of progeny generated are less than might have been expected. Nevertheless in one year 107 KI× F1 offspring (Table 3.3) and in two years 85 F1 or backcross × parental offspring (Table 3.1) were born, potentially allowing detection of linkage between genes which are up to 40cM and 39cM apart respectively. Therefore sufficient numbers of test cross progeny have been bred for the efficient generation of a linkage map of the tammar wallaby genome. Whilst no difficulties in mating occur at this level, one must

also consider how readily WA and SA subspecies hybridise as the generation of F1 hybrids is the first step to breeding offspring for the test cross panel.

The separation of the Australian mainland from its offshore islands is a relatively recent event, brought about by the rise in sea levels after the last Pleistocene ice age ended about 10,000 years ago (Kennedy 1992). It follows that the WA populations of tammars have been geographically isolated for a short time in evolutionary terms. Whilst the time of separation between the WA populations may not be great, the length of geographical isolation between them and the SA population could be between 50,000 to 100,000 years ago (Oliver et al., 1979; N. McKenzie pers. comm.). In this thesis WA and SA tammars are referred to as subspecies. It is, however, debateable as to whether SA and WA animals are subspecies or different species (Poole et al., 1991, van Oorschot and Cooper, 1988a). Species are usually recognised by assessment of morphological and physiological differences and the extent of reproductive isolation. Defining species on the basis of these features is not always clear and in these cases inclusion of information regarding their genetic distance (D) can be useful.

There are subtle morphological differences between tammar populations. For example, GI animals are rufous in colour and AI animals have a smaller head to body ratio. (It must be said that whilst these differences would mostly be noticed by the experienced - to the casual observer a tammar would look like a tammar). Morphometric analysis of crania suggest that there are distinct differences between the SA and WA populations (Poole et al., 1991).

A small number of observations have been made regarding physiological features of the WA populations. It is clear from these observations that, at least in the case of MI animals, there are quite distinct differences. MI tammars do not have the same breeding season as some other tammar subspecies. Reactivation of the dormant blastocysts can occur before the summer solstice (22nd December) in MI females. The earliest birth recorded at Macquarie was on 7th November, 1993. Two births occurred in December 1993 and three births in early January, 1994. It has been

reported that hybrid kangaroo fetuses are born after gestation periods intermediate between that of the parental genotypes (Kirsch and Poole 1972; Poole 1975). This was not seen in KI×MI hybrids which were all born after January, 1994 (data not shown). The data set is very small, only 4 animals, as this mating has not been successful in past years (Tables 3.3 and 3.4).

The relationship between genetic distance and subspecies/species relationships is not clear cut. For example, the D between ten subspecies of pocket gophers ranges from 0.004 to 0.262 and the D between six species of Macaques ranges from 0.02 to 0.10 (reviewed in Nei 1987). It has been proposed by Thorpe (1982) that in cases where genetic distance data is used to clarify subspecies/species relationships that allopatric populations with a $D > 0.16$ are unlikely to be conspecific and that a genetic distance of < 0.16 suggests that, in the absence of other evidence, the populations should be considered subspecies. The Nei genetic distance between GI and KI tammars is 0.16 calculated on allozyme and protein data (this analysis is discussed in Section 4.3.1). This figure is consistent with the GI and KI populations being either distant subspecies or closely related species. There are no data regarding the genetic distance between KI and any other WA population, or between the WA populations themselves.

The last criterion for determining subspecies/species status is the degree of reproductive isolation. One of the first signs of a genetic barrier to reproduction is infertility in male F1 hybrids and backcrosses (Haldane, 1922). There is no significant difference between the fertility rates of "pure" subspecies, F1 or backcross male animals (Table 3.5.; $\chi^2_2 = 2.05$, $p > 0.05$). There is, however, evidence to support the suggestion that a barrier to reproduction exists when crossing SA with WA animals to produce F1 hybrids. Table 3.5 shows that the BE for KI×WA crosses is approximately 1/3 of the BE for other crosses. Fewer data were available for WA× KI crosses, however the BE for these matings is approximately 2/3 of the BE for other crosses. Examination of the BE for KI females run with GI bucks over sequential years indicates that it increased in the second year of running the

subspecies together (Table 3.6). Anecdotal evidence supports these conclusions. Observations were made at CSIRO Division of Wildlife and Ecology that, at least in the first season of running subspecies together, there is restricted mating. In a yard containing KI and GI females and a GI buck, the KI females ran several cycles without attracting the attention of the GI male despite interest from KI males in neighbouring yards. The barrier appeared to lessen in subsequent years (WE Poole and N Simms pers. comm.).

Two hypotheses for this trend are suggested:

i) Specific fertility of the male. For example, in 1992, T75 (GI male) was run with KI females and two KI×GI females, none of which reproduced (Table 3.6). T75 was found dead in August, 1992. None of the females run with T75 gave birth in 1993 as a result of postpartum mating in 1992. It is not possible to determine whether lack of births was a result of reproductive isolation or other unknown factors such as reduced male fertility.

ii) Increased attention of the GI male or increased acceptance by KI females. For example, 389 (GI male) was run in a yard holding only KI females during 1992 and 1993 (Table 3.6). The data suggests that there was a higher efficiency of pouch young production in the second year.

More data is required from specifically designed matings in order to clarify the origin of these apparent behavioural barriers to reproduction.

That some degree of reproductive isolation exists between WA and SA tammar populations is supported by the data in Table 3.5. There are significant differences in the BE between the different types of crosses ($\chi^2_6 = 76.86$, $p \leq 0.05$). However, once crosses designed to produce F1 hybrid progeny (KI×WA and WA×KI) are removed from the analysis, no heterogeneity is detected ($\chi^2_4 = 7.44$, $p \geq 0.05$). The reduced BE between WA and SA subspecies is illustrated by the failure to produce any KI×MI F1 hybrids during 1993. This is consistent with there being a behavioural barrier to reproduction but could well be complicated by the differences in the reproductive cycle of the two subspecies. It would be of great interest to

attempt the reverse cross and also to attempt matings between other WA populations (which appear to have the same breeding cycle as KI tammars) and MI animals.

The number of animals used at Macquarie was too small to allow consideration of the possibility of preferential mating in those yards where a particular subspecies of buck eg. KI, was run with both SA and WA females. In order to examine this question more closely it would be necessary to run sufficient numbers of SA and WA tammars in an area large enough to allow for the formation of distinct territories.

There are many other factors which could affect breeding success which were not controlled for but must be considered in the interpretation of the data. A number of animals used in this study, particularly the WA subspecies, were caught in the wild (W. Poole and N. Simms, pers. comm.) Hence, important information for assessing and comparing success of breeding such as the age of the animal, parity and specific fertility rates, are unknown. This problem is exemplified by attempts to cross GI females with KI males. During 1992 three GI females, originally collected from Garden Island were run with one KI buck, E104. No progeny were produced. Another GI female from Garden Island was run with a GI buck and had a pouch young. In 1993 these four GI females were run with E104. Three pouch young were born but none survived past three months. It is not possible to determine whether the lack of F1 hybrid progeny is a result of age of the females, age of the male, failure to mate, failure to conceive, reduced pouch young survival rate or even conditions of the yard in which they were run. There is no doubt, however, that F1 hybrids of this genotype are possible. One female and four males have been produced at CSIRO Division of Wildlife and Ecology between 1987 and 1990 (WE Poole pers. comm.).

It must also be stressed that this breeding program was undertaken in a captive situation. Macquarie University Fauna Park is divided into 52 separate enclosures. The size of yards and numbers of animals held in each yard varied. As well as variable size, there is also variation in the degree and type of shelter available in each yard. Some have man-made wooden shelters or concrete tubing, others have

low scrub and some have very little shelter of either kind. The natural habitat of tammar wallabies is dry sclerophyll forest, heathlands, coastal scrub, mallee and woodland thickets (Kennedy 1992). It has become clear that breeding success is influenced, at least to some degree, by the availability of appropriate shelter. One example was seen in attempts to breed MI animals. In 1993 seven females were run with one buck in a large yard which had very little shelter. Three pouch young were born during the breeding season, all of which were lost. These animals were then moved to another yard, 50% of which was covered by low scrub. By March 1994 six out of seven females were carrying pouch young (data not shown).

A controlled experimental environment involving animals of known age, parity and specific fertility rates, standardised yard size, available shelter and catching regime, and sufficient area for any possible selective mating to occur, would be necessary to answer fully the question "is there any barrier to reproduction between SA and WA tammars?". The observations presented here do indicate strongly that there is, at least, a behavioural barrier to reproduction between WA and SA tammar wallaby subspecies. Whether the reduced efficiency of producing F1 hybrids is due only to reduced mating is not clear and further research is required. Observations of behaviour and the efficiency of producing pouch young suggest that this barrier does not extend to any further crosses. Hence, the limiting step in generating test cross progeny from subspecies crosses for mapping the tammar wallaby genome through recombination is the production of F1 hybrids. Macquarie now holds a considerable number of male and female hybrids, and the production of test cross progeny is now as efficient as the generation of "pure" subspecies offspring.

The debate will continue regarding the specific status of different tammar populations. They are morphologically and physiologically distinct in some respects, the genetic distance between KI and GI is supportive of them being either distantly related subspecies or closely related species, and the beginnings of reproductive isolation between them appears likely.

Note: observations from 1990 were included for completeness. I was not involved with this work at that time.

Table 3.1. Number of pouch young included in panels of test cross progeny for linkage studies born to female F1 hybrid and backcross tammars during 1992 and 1993.

		1992			1993		
Female	Genotype	Male	Genotype	No. progeny	Male	Genotype	No. progeny
T139	KIxGI	E6462	KI	2	E6462	KI	2
T102	KIxGI	E1234	KI	2	E6462	KI	2
T73	KIxGI	E1234	KI	4	E1234	KI	1
T76	KIxGI	E1234	KI	3	E6462	KI	2
T120	KIxGI	339	GI	2	---	---	---
T119	KIxGI	339	GI	2	339	GI	1
T134	KIxGI	349	GI	4	339	GI	1
T113	KIxGI	349	GI	1	349	GI	2
T81	KIxGI	349	GI	3	339	GI	1
T108	KIxGI	349	GI	4	349	GI	1
T98	GIxKI	E1234	KI	4	E1234	KI	2
E1021	GIxKI	E1234	KI	5	E6462	KI	2
T115	KIx(GIxKI)	E6462	KI	2	E6462	KI	3
T125	KIx(GIxKI)	E6462	KI	2	E6462	KI	1
E1083	KIx(GIxKI)	E6462	KI	3	E6462	KI	2
E1034	KIx(GIxKI)	E6462	KI	2	E6462	KI	3
E1084	KIx(GIxKI)	E6462	KI	4	---	---	---
T196	KIx(GIxKI)	E1234	KI	4	E1234	KI	3
E1017	KIx(KIxGI)	E6462	KI	2	---	---	---
E1019	KIx(KIxKI)	E6462	KI	1	---	---	---
	TOTAL			56			29
	MEAN PER FEMALE			2.8			1.8

KI = Kangaroo Island, GI = Garden Island.
The maximum each female could produce was four to five offspring per year.

Table 3.2. Number of progeny born per possible oestrous cycle for all tammar wallabies by year.

Year	Total no. possible oestrous cycles	Non-fertile	Fertile	BE
1990	163	121	42	25.8
1991	216	97	119	55.1
1992	312	185	127	40.7
1993	215	126	89	41.4
Total	906	529	377	41.6

BE = Breeding Efficiency

Table 3.3. Breeding data for KI, GI, MI, AI, P, F1 and backcross tammar wallabies in 1990-1993.

Year	Mother	No. of Females	Father	No. of Males	No. possible oestrus cycles	No. pouch young
1990	KI	17	KI	2	32	12
	KI	11	GI	1	26	5
	KI	21	KI×GI	1	54	10
	KI	24	GI×KI	1	51	15
1991	KI	11	KI	1	20	8
	GI	4	GI	5	7	3
	KI	4	GI	5	13	1
	KI	11	KI×GI	1	44	27
	KI	33	GI×KI	3	132	80
1992	KI	15	KI	3	31	19
	GI	9	GI	2	29	10
	KI	21	GI	5	79	9
	GI	4	KI	2	13	4
	AI	1	GI	1	3	1
	KI×GI	4	KI	2	16	11
	KI×GI	6	GI	2	24	16
	GI×KI	2	KI	1	4	4
	GI×KI	4	GI	1	4	4
	KI×AI	1	KI	1	4	1
	MI×AI	1	GI	1	3	1
	KI×(GI×KI)	6	KI	2	28	20
	KI×(KI×GI)	4	GI	1	8	3
	KI	12	KI×GI	1	48	14
	KI	6	KI×(KI×GI)	2	9	5
	KI	6	KI×(GI×KI)	2	9	5
1993	KI	12	KI	1	36	27
	GI	4	GI	1	10	2
	P	3	P	1	8	6
	KI	17	GI	2	41	15
	GI	4	KI	1	8	3
	KI	7	MI	1	28	0
	KI	1	P	1	4	0
	KI	12	KI×GI	1	12	6
	KI×GI	4	KI	2	16	7
	KI×GI	5	GI	2	20	6
	GI×KI	2	KI	2	8	4
	KI×(GI×KI)	5	KI	2	24	13

KI = Kangaroo Island, GI = Garden Island, MI = Middle Island, AI = Abrolhos Group, P = Perup.

Table 3.4. BE among KI, GI, AI, MI, P, F1 and backcross tammar wallabies for 1990-1993 combined.

Mother	No. females	Father	No. males	No. possible oestrous cycles	No. pouch young born	BE
KI	55	KI	7	119	66	55.5
GI	17	GI	8	46	15	32.6
P	3	P	1	8	6	75.0
KI	53	GI	13	159	30	18.9
GI	8	KI	3	21	7	33.3
KI	1	P	1	4	0	0.0
KI	7	MI	1	28	0	0.0
AI	1	GI	1	3	1	33.3
KI	56	KI×GI	4	158	57	36.1
KI	57	GI×KI	4	183	95	51.9
KI×GI	8	KI	4	32	18	56.2
KI×GI	11	GI	4	44	22	50.0
GI×KI	4	KI	3	12	8	66.7
GI×KI	4	GI	1	4	4	100.0
KI×AI	1	KI	1	4	1	25.0
MI×AI	1	GI	1	3	1	33.3
KI×(GI×KI)	11	KI	4	52	33	63.5
KI×(KI×GI)	4	GI	1	8	3	37.5
KI	6	KI×(KI×GI)	2	9	5	55.5
KI	6	KI×(GI×KI)	2	9	5	55.5

KI = Kangaroo Island, GI = Garden Island, MI = Middle Island, AI = Abrolhos Group, P = Perup.
BE = Breeding Efficiency

Table 3.5. The BE of parental subspecies, F1 hybrids, first backcrosses and second backcrosses.

Cross	No. possible oestrous cycles	Fertile	Non-fertile	BE
Within subspecies	173	87	86	50.3
KI×WA	191	30	161	15.7
WA×KI	21	7	14	33.3
F1×Parental	96	53	43	55.2
Parental×F1	341	152	189	44.6
Backcross×Parental	60	36	24	60.0
Parental×Backcross	18	10	8	55.5

KI = Kangaroo Island, WA = Western Australia.
BE = Breeding Efficiency

Table 3.6. Number of pouch young born per possible oestrous cycle to KI females run with GI bucks.

		1991			1992			1993	
KI female	GI male	No. possible oestrous cycles	No. pouch young	GI male	No. possible oestrous cycles	No. pouch young	GI male	No. possible oestrous cycles	No. pouch young
E6658	multiple	4	0	multiple	2	2			
E1003	multiple	4	0	multiple	4	0			
E650	multiple	4	0	multiple	4	1			
E6655	multiple	1	1	multiple	4	1			
E1002				389	2	1	389	2	1
E201				389	3	1	389	2	1
E7050				389	4	0	389	2	1
E623				389	4	0	389	4	0
E6351				389	4	0	389	2	1
E6750				339	4	2	349	2	1
E6480				339	4	1	349	1	1
E7058				T75	4	0	349	1	1
E1075				T75	4	0	349	2	1
E7524				T75	4	0	349	2	1
E6660				T75	4	0	349	3	1
E4				T75	4	0	349	4	2
E1037				T75	4	0	349	4	0

KI = Kangaroo Island, GI = Garden Island.
Only females run with a GI male for more than one year are included.

CHAPTER 4

GENETIC VARIATION IN THE TAMMAR: IMPLICATIONS FOR CONSERVATION

4.1 INTRODUCTION

Extant marsupials are confined to the Australian biogeographical region (Australia, New Guinea, Irian Jaya and Sulawesi) and the Americas (Kirsch and Calaby 1977). There are about 250 species of which two-thirds are from the Australian region where they are the dominant group of mammals. The distinctiveness of the Australian fauna results from its long isolation from the faunas of other land masses since separation from the supercontinent of Gondwana early in the Tertiary, some 45 million years ago. Australia's distinctiveness does not end here. Australia has the worst extinction rate for mammals of any continent or country and accounts for about half the world's mammals that have become extinct in modern times. Ten Australian marsupial species are known to be extinct and 19% of extant species are currently listed as vulnerable or in danger of extinction (Burbidge and N. McKenzie 1989; CONCOM 1991). The most likely causes of extinction or decline in species numbers have been identified as i) the introduction of exotic herbivores such as rabbits, goats, cattle and sheep, ii) altered fire regimes, and iii) the introduction of effective predators such as foxes and feral cats (Kennedy 1992).

Australia's 160 offshore islands have proved to be immensely valuable in providing refuge for some critically threatened species. Thirty one species listed as endangered, vulnerable or potentially vulnerable to extinction occur on offshore islands, including the tammar. The tammar's former range included semi-arid areas of the southwest of WA and SA, five WA islands and seven SA islands. Tammars are now extinct on the SA mainland and six of the seven SA islands, although it is not clear whether the populations on three of these islands (St Francis, Reevesby and Wedge) were natural or the result of introductions by sailors in the 18th and 19th

centuries. The SA government introduced tammar wallabies to Granite and Boston Islands, which were outside the species' historical range, during the 1970's. Tammars have now been removed from Granite Island by the government. Tammars were also introduced to Greenly Island by fishermen at the turn of the century (Mitchell and Behrndt 1949) (Table 4.1). Introductions outside of Australia have occurred, notably to Kawau Island, New Zealand in 1870 by Sir George Grey and to the Rotorua region of the North Island about 1912. The only known remaining Australian mainland population of tammars occurs in the Perup region of southwest WA, the area with the greatest concentration of endangered or vulnerable marsupial species in the continent. Along with many other species from this region, the numbers of "Perup" tammars have declined dramatically in recent years. Australia's offshore islands will probably prove to be the last refuge for the tammar wallaby in its native habitat.

Small, isolated populations of mammals, like those confined to islands, are particularly vulnerable to loss of genetic variability due to random genetic drift as well as other stochastic events such as disease, fire and predation (George and Brown 1992). As tammar numbers decline on the mainland, it is of great importance to assess the genetic variability of extant island populations. To this end, animals from GI and KI, were typed for 54 genetic markers. The data were used to calculate percent polymorphism, heterozygosity and average percent difference within each subspecies. Although KI is an island, it is of sufficient size (450,000 ha) to assume that this is a population which is not under pressures of a "typical" island situation. Thus, the first aim of this work was to assess whether there is any difference in the level of heterozygosity or other measures of genetic variability between the two populations.

It has become clear that there is an additional complication that must be considered when contemplating conservation management of the tammar wallaby. Island populations have been isolated from each other and the mainland for about 10,000 years, after the rise in sea level caused by the ending of the Pleistocene ice age

(Kennedy 1992). The WA and SA populations have been isolated from each other for between 50,000 and 100,000 years (Oliver et al., 1979; N. McKenzie pers. comm.). These populations are morphologically (Poole et al, 1991) and physiologically distinct (discussed in Chapter 3) and the beginnings of reproductive isolation between them seems likely (Chapter 3). Previous studies of allozyme and protein polymorphisms have shown that the populations are also genetically distinct (van Oorschot and Cooper 1988a; van Oorschot et al, 1989; van Oorschot 1989). It is possible that some of the different island populations collectively known as *Macropus eugenii* could be different species. If this is the case, it is of great importance to manage each population individually. The data presented here thus enable a second question to be addressed regarding the genetic relationship between SA and WA populations.

4.2 MATERIALS AND METHODS

4.2.1 Animals

See Chapter 3 for a discussion of animal breeding and husbandry.

4.2.2 Allozyme Electrophoresis

Allozyme electrophoresis was performed on red blood cell lysates and liver samples according to Richardson et al. (1986) for 29 markers which are listed in Table 4.2.

4.2.3 Isoelectric Focusing

Isoelectric focusing (IEF) for the serum proteins ALB (albumin), Pi (protease inhibitor), TF (transferrin) and plasma esterase were carried out according to the

technique of Righetti (1983). Ampholines of the pH ranges pH = 3.5-5, pH = 5-7 and pH = 7-9 were mixed in equal quantities and used.

4.2.4 DNA Extraction and Southern Hybridisation

Genomic DNA was extracted from blood and tissue according to Sambrook et al. (1989), except that tissue was initially homogenised in 1ml of 2mM $CaCl_2$, 50mM Tris (pH 8.0) and 200ug of proteinase K for 1hr at 65°C. 7ug of DNA was digested with various restriction enzymes (Boehringer Mannheim or Promega, Australia) and electrophoresed on 0.8% agarose gels. DNA was transferred to Hybond N membranes (Amersham) and hybridised with ^{32}PdATP labelled probes. The 22 DNA probes used for Southern hybridisation are described in Table 4.3.

4.2.5 Statistics

Estimates were made for mean average percent difference (MAPD), average heterozygosity (H_A), percent polymorphism and genetic distance (D). These values were calculated according to Yuhki and O'Brien (1990), Gilbert et al. (1990), Stephens et al. (1992) and Nei (1987) respectively.

4.3 RESULTS

The numbers of KI and GI tammars typed for allozyme, protein and restriction fragment length polymorphism (RFLP) markers are presented in Tables 4.4 and 4.5. Forty three markers identified a single locus and six markers identified two to four loci. There was insufficient data to calculate the exact number of loci detected by some cDNA probes. In these cases the least number of fragments revealed by a probe was taken as the lower limit for the number of loci detected. Three markers detected multiple loci and one marker was mitochondrial (Table 4.6).

4.3.1 Allozyme and Serum Protein Markers

KI and GI tammar wallabies were screened for 28 allozyme and four serum protein markers in order to identify intrapopulation and interpopulation

polymorphisms (Table 4.5). Twenty three markers were invariant, four were polymorphic in KI only, one was polymorphic in GI only, one was polymorphic in both and four revealed a fixed difference between the two populations (Table 4.7). The average heterozygosity (H_A) within KI and GI was 1.078 and 0.933 respectively (Table 4.5). The genetic distance (D) between the two populations was calculated to be 0.16. (Calculations were by D.W. Cooper; genetic typing was by Cooper and Bell, unpublished data; van Oorschot and Cooper, 1988a).

4.3.2 RFLP Markers

DNA from KI and GI tammar wallabies was digested with one to seven different restriction enzymes and hybridised with 22 DNA probes. The numbers of monomorphic and polymorphic fragments detected by each probe/enzyme combination are listed in Table 4.8. Those probe/enzyme combinations which revealed fixed differences between KI and GI are also shown. The number of markers which were invariant, polymorphic or revealed fixed differences are summarised in Table 4.7.

The proportion of polymorphism detected by each probe against a suite of enzymes (P_e) or each enzyme against a suite of probes (P_l) are listed in Table 4.9. This table only includes data generated by probes which were known to hybridise to a single locus, as it was not possible to determine conclusively how many loci were detected by other probes and, consequently, the proportion of these loci which were polymorphic. χ^2 analysis for heterogeneity indicated that there was no significant differences between P_e in KI ($\chi^2_{14}=21.31$, $p > 0.05$) or GI ($\chi^2_{14}=24.45$, $p > 0.025$) or P_l in KI ($\chi^2_7=2.56$, $p > 0.05$) or GI ($\chi^2_7=4.07$, $p > 0.05$). There was also no significant difference between the overall proportion of polymorphism when comparing the KI and GI populations ($\chi^2=0.06$, $p > 0.05$).

The mean average percent difference (MAPD) for all probe/enzyme combinations tested was calculated to be 4.40% and 3.36% for KI and GI respectively (Table 4.10).

H_A for all probe/enzyme combinations was calculated to be 0.0962 and 0.0959 for KI and GI respectively (Table 4.11). H_A calculated on the basis of polymorphic loci only was 0.3038 and 0.3614 for KI and GI respectively.

The average number of KI and GI individuals typed for RFLP markers was 14.86 and 7.78 respectively and allozyme or protein markers was 60.6 and 17.0 respectively. These data along with an overall summary of the level of genetic variability within each population are presented in Table 4.12. It is clear from this table that whilst there is a slight trend for GI values to be lower than KI values, the differences are not significant and may well be a reflection of the numbers of GI animals sampled.

4.4 DISCUSSION

In order to assess the effects of isolation and small population size on tammars, KI and GI animals were typed for 54 genetic markers. No significant differences were observed in the proportion of polymorphism, MAPD, H_A based on RFLPs or H_A based on allozymes and proteins, between the two populations.

Population genetics theory predicts that small, isolated populations will undergo a loss of heterozygosity due to genetic drift, in comparison to a larger population. Given that GI has been separated from the mainland for about 10,000 years (Kennedy 1992) and assuming a mean generation time for tammars of between 3 and 5 years, we can estimate that this population has been isolated for about 2,000 - 3,000 generations. Loss of heterozygosity is dependent on the effective population number (N_e). The heterozygosity (H_t) of a population at time (t) is given by the equation

$$H_t/H_o = (1- 1/2N_e)^t$$

where H_t is the level of heterozygosity at t = 0 (Crowe and Kimura 1970; Nei 1987). Table 4.13 lists the level of H_t over different numbers of generations for various

values of N_e. A provisional estimate of 200 as the maximum effective population number on GI was made on the basis of the area of the island. The predictions made by this equation suggest that a population with an N_e of 200 will have lost 99.3% heterozygosity after 2000 generations (Table 4.13). Surprisingly, the level of genetic variation in GI tammars is not significantly different to that seen in the much larger KI population. There are several possible explainations as to why KI and GI tammars have such similar levels of heterozygosity.

i) The N_e on GI could be greater than 200. A survey of the actual numbers of tammars on GI and studies of their social structure and breeding behaviour will be important in the clarification of this question. An N_e of 500 would, in approximate terms, require that there be one reproductively active male every 400 m^2. This would seem to be the upper limit of density of tammars on the island. Even if the N_e of GI was 500, the population would be expected to have lost approximately 87% of its heterozygosity after 2000 generations (Table 4.13).

This is even more surprising considering that comparisons of similar RFLP data available for eutherian species with data presented here indicates that levels of heterozygosity are not significantly different for tammar wallabies, sheep and humans. The calculated H_A for a flock of merino sheep has been reported to be 0.1050 (Parsons 1994). This is not significantly different to the H_A of KI and GI (0.0962 and 0.0959 respectively). Data presented in Table 4.9 was further analysed after removal of monomorphic loci to allow for comparison with sheep and humans. The H_A based on polymorphic loci only was 0.385 for sheep (Parsons 1994) and 0.325 (based on 73 loci) for humans (Bowcock et al., 1991). Again, these values do not differ significantly from calculations of the H_A based on polymorphic loci for KI and GI tammars (0.3038 and 0.3614 respectively).

ii) The N_e of GI could approximate the N_e of KI. Without a reliable estimate of the numbers of animals on each island, this possibility cannot be eliminated. It seems unlikely, however, as KI is about 430 times larger than GI (Table 4.1).

iii) GI might not have been isolated from the mainland for 10,000 years as predicted by the known time of the rise in sea level. As tammar specimens have been found on the mainland near GI (Poole et al., 1991) it could be that the population, until relatively recently, was much larger. Further geological information on the actual time of isolation of GI is needed.

iv) Selection may be acting. Whilst this possibility must be considered, the number and randomness of loci examined in this study suggests that this is an unlikely explanation.

A survey of the numbers and reproductive behaviour of tammars on islands will enable a more stringent interpretation of the surprising result that there is no significant difference between the level of genetic variability in KI and GI tammar wallabies. Similar genetic analysis of other island populations, particularly North Twin Peaks which is approximately 25% of the size of GI, would enhance our knowledge of the effects of isolation on small populations of these macropodids.

That GI tammars have similar levels of genetic variability to KI tammars or to those found in humans and sheep was unexpected. The various sizes of island populations of tammars and the growing body of literature regarding the genetics of some of these populations provides a unique opportunity to study the effects of isolation of small populations of these macropodids.

There is another important aspect of the data which pertains to strategies for conservation management of tammar wallabies. The SA and WA populations could have been geographically isolated for between 50,000 and 100,000 years (Oliver et al., 1979; N. McKenzie pers. comm.). The biology of the KI population of tammar wallabies has been studied intensively over the last decade and as such is now considered a reference model for macropodid species (Hinds et al., 1990). Most advances in understanding have pertained to its reproductive cycle and genetics. Whilst there is an increasing body of literature for this, the only SA population of tammars, relatively little is known of the WA populations. The small number of

comparative investigations between SA and WA populations suggest that there are significant differences in their biology (Poole et al., 1991; Cooper and van Oorschort, 1988a; van Oorschot, 1989, WE Poole pers. comm.). Chapter 3 describes breeding data between WA and SA tammars which shows the probable beginnings of reproductive isolation between them. Whether they should be regarded as widely distant subspecies or very closely related species would reflect the definition of a species preferred by the person making the judgement.

The need for further studies of the taxonomy, behaviour and genetics of tammar wallaby populations is clear. Such studies could lead to the relisting of some populations from "vulnerable to extinction" to "endangered" or "extinct". The genetic distance between the KI and GI populations would indicate that any programs designed to reintroduce tammars to historical ranges, or to increase numbers in dwindling mainland populations, must take into account the genetic diversity of what is now, perhaps incorrectly, considered one species.

Table 4.1. Former and current distribution of Australian island populations of tammars.

ISLAND	AREA (ha)	STATE	COMMENTS
Flinders	4-5,000	SA	Extinct
St. Peter	approx. 4,000	SA	Extinct
Thistle	4,113	SA	Extinct
St. Francis*	809	SA	Fossil remains only
Reevseby*	approx. 500	SA	Fossil remains only
Wedge*	967	SA	Fossil remains only
Boston	967	SA	Introduced 1971
Granite"	32	SA	Introduced 1970's
Greenly	141	SA	Introduced 1905
Kangaroo	450,000	SA	Extant
East & West Wallabi^	894	WA	Extant
Garden	1,054	WA	Extant
Middle	1,036	WA	Extant
North Twin Peaks	272	WA	Extant

Adapted from Kennedy, 1992.

SA=South Australia; WA=Western Australia
* it is unclear whether these were naturally occurring populations or the result of introductions by sailors in the 18th and 19th centuries.
" this population has been removed (SA Tourist Bureau pers. comm.)
^ East and West Wallabi Islands, part of the Abrolhos group, are connected at low tide. Tammar wallabies move between the two (WE Poole pers. comm.), and as such are considered one area for the purposes of this discussion.

Table 4.2. List of allozyme markers used in genetic typings of tammars.

ACO1	Aconitate hydratase 1
ADA1	Adenosine deaminase 1
AK1	Adenylate kinase 1
AK2	Adenylate kinase 2
ALDOH	Aldolase
ENO	Enolase
ESD	Esterase D
FH	Fumarate hydratase
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
GLA	α-galactosidase
GLO	Lactoyl-glutathione lyase
GOT	Asparate aminotransferase
GPD	Glucose-6-phosphate dehydrogenase
GPI	Glucose-phosphate isomerase
GPT	Alanine aminotransferase
HK	Hexokinase
IDH1	Isocitrate dehydrogenase 1
MDH1	Malate dehydrogenase 1
ME1	Malic enzyme 1
ME2	Malic enzyme 2
MPI	Mannose phosphate isomerase
NP	Purine nucleoside phosphorylase
PGD	6-phosphogluconate dehydrogenase
PGK	Phosphoglycerate kinase
PGM1	Phosphoglucomutase
PK	Pyruvate kinase
SOD	Super oxide dismutase
SORDH	L-iditol dehydrogenase

Table 4.3. DNA probes used for Southern hybridisations.

Probe	Locus	Chromosome location	Species	Vector	Insert size (kb)	Cloning site	# loci detected	Source
pG115		A	M.e.	pGem4	2.3	Eco RI	1	C.C.
pG1211	LALBA	A	M.e.	pGem4	0.8	Eco RI	1	C.C.
pG862	LLP	3	M.e.	pGem4	0.8	Eco RI	1	C.C.
pB22	anon	A	M.e.	pGem4	2.7	Eco RI	1	C.C.
pB65	anon	3	M.e.	pGem4	2.0	Eco RI	1	C.C
pB61	anon	A	M.e.	pGem4	3.5	Eco RI	1	C.C.
pB54	anon	A	M.e.	pGem4	3.0	Eco RI	1	C.C
pB3	LPL	3	M.e.	pGem4	1.0	Eco RI	1	C.C.
pB15	anon	A	M.e.	pGem4	0.8	Eco RI	1	C.C.
pB12	anon	A	M.e.	pGem4	1.9	Eco RI	1	C.C.
pB67	anon	A	M.e.	pGem4	1.6	Eco RI	≤3	C.C.
pB 72	anon	A	M.e.	pGem4	2.0	Eco RI	1	C.C.
pG1333	anon	A	M.e.	pGem4	0.6	Eco RI	≤4	C.C
rRNA	RNR	A	Xen	pGem4	12.0	Hind III	1	K.J.
HPRT 4.1	HPRT	Xq	M.r.	pUN121	4.1	Hind III	1	A.P.
Ex 5-13*	GPD	Xq	M.r.	------	1.2	-----	1	P.J.
pG777	CASA	A	M.e.	pGem4	1.1	Eco RI	≤3	C.C
pG848	CASB	A	M.e.	pGem4	1.2	Eco RI	1	C.C.
Tf34*	anon	A	M.e.	------	0.6	-----	≤2	T.F.
pG1050	COX-1	M	M.e.	pGem4	1.6	Eco RI	1	C.C.
Maru-DBB	MHC II	A	M.ru.	M13	1.1	Hind III	≥12	S.S.
ME5	PGK	A, X	M.e.	pGem4Z	1.3	Eco RI /Xba I	25-30	T.F.

* these probes are uncloned PCR products
LALBA = α-lactalbumin, LLP = late lactation protein, LPL = lipoprotein lipase, GPD = glucose-6-phosphate dehydrogenase, CASA = α_1-casein, CASB = β-casein, COX -1 = cytochrome oxidase C, MHC II = major histocompatibility class II sequence, PGK = phosphoglycerate kinase
A = autosomal, M = mitochondrial
M.e. = *Macropus eugenii*, M.r. = *Macropus robustus robustus*, M.ru. = *Macropus rufogriseus*, Xen = *Xenopus*
C.C. = Chris Collet, K.J. = Ken Jones, A.P. = Anita Piper, P.J. = Peter Johnston, T.F. = Tali Feferman, S.S. = Susan Schneider.

Table 4.4. Number of KI and GI tammars typed for each probe/enzyme combination.

	Kangaroo Island								Garden Island							
R E / PROBE	T	Ba	Pv	E	B	H	P	M	T	Ba	Pv	E	B	H	P	M
pG115		6	6	6	6	6				6	6	6	6	6		
pG1211	5	5	5	5	46	5	5		5	5	5	5	20	5	5	
pG862	46			14			14		5							
pB22	5	5	5	5	5	5	5		5	5	5	5	5	5	5	
pB65	38	5		5	5	5			5	5		5	5	5		
pB61	5			5	5	41			5			5	5	4		
pB54		5	5	5	5	5				5	5	5	5	5		
pB3				5	5	35	5					5	5	5	5	
pB15	5	5			46	5			5	5			5	5		
pB12					46	5							20	5		
pB67	5				5	5			5				5	5		
pB72	22				33				7				20			
pG1333	5				5				5				5			
rRNA			6	6	6	6					6	6	6	6		
HPRT4.1	5		5		5				5		5		5			
Ex5-13	3					5			3					5		
pG777	134		11		11			4	4		11		11			3
pG848	5		5		5	5		5	5		5		5	5		5
pG1050	5		11		11	11			5		11	10	11			
Tf34						5								5		
DBB	5		5		5	5			5		5		5	5		
ME5	41		3			6		3	11		4			11		4

R E=Restriction Enzyme; T=Taq I; Ba=Bam HI; Pv=Pvu II; E=Eco RI; B=Bgl II; H=Hind II; P=Pst I; M=Msp I
KI = Kangaroo Island; GI = Garden Island

Table 4.5. Calculation of genetic distance and heterozygosity in KI and GI tammars for allozymes and serum proteins.

LOCUS	ALLELE	KI	GI	H_K	H_G	No. KI	No. GI
ACO 1	A	1	1	0	0	24	1
ADA 1	A	1	1	0	0	50	25
AK 1	A	1	1	0	0	29	22
AK 2	A	1	1	0	0	10	20
ALDOH	A	1	1	0	0	12	3
ENO	A	1	1	0	0	18	22
ESD	A	0.99	1	0.02	0	204	19
	B	0.01	0				
FH	A	1	1	0	0	18	3
GAPD	A	1	1	0	0	18	3
GLA	A	1	1	0	0	1	1
GLO	A	1	1	0	0	19	22
GOT	A	1	1	0	0	34	4
GPD	A	1	1	0	0	74	20
GPI	A	0.027	0	0.054	0	245	34
	B	0.973	1				
GPT	A	1	1	0	0	18	3
HK	A	1	1	0	0	18	7
IDH 1	A	1	1	0	0	23	20
MDH 1	A	1	1	0	0	32	4
ME 1	A	1	1	0	0	28	4
ME 2	A	1	1	0	0	27	1
MPI	A	1	0	0	0	33	26
	B	0	1				
NP	A	0	1	0	0	44	25
	B	1	0				
PGD	A	0.034	0	0.068	0	133	37
	B	0.966	1				
PGK	A	1	1	0	0	152	26
PI	I	0.097	0	0.420	0	193	26
	M	0.238	0				
	P	0.065	0				
	L	0	1				
PGM 1	A	1	1	0	0	25	20
PK	A	1	1	0	0	2	19
SOD	A	1	1	0	0	224	26
SORD	A	1	1	0	0	18	3
TF	A	1	0	0	0	100	23
	B	0	1				
EST	A	0.081	0.186	0.516	0.370	93	59
	A'	0.129	0	0.167			
	B	0.650	0				
	C	0.086	0				
	D	0.054	0				
	E	0	0.814				
ALB	A	1	0.590	0	0.563	20	16
	B	0	0.410				

(Cooper and Bell, unpublished data; van Oorschot and Cooper, 1988)
KI = Kangaroo Island; GI = Garden Island
H_K and H_G = Average heterozygosity for KI and GI tammars respectively
No. KI and No. GI = Numbers of KI and GI animals typed respectively

Table 4.6. Estimates of the number of loci detected by allozyme, protein and DNA markers used for typing the KI and GI populations of tammars.

	Allozyme	Protein	DNA probe	Total
single locus	25	3	15	43
two loci	2	0	0	2
≤ two loci	0	0	1	1
≤ three loci	0	0	2	2
≤ four loci	0	0	1	1
multilocus	0	1	2*	3
mitochondrial	0	0	1	1

*ME5 (phosphoglycerate kinase) detects 25-30 loci (Cooper et al., 1994); DBB (major histocompatibility class II) detects approximately 12 loci (Chapter 6).

KI = Kangaroo Island; GI = Garden Island

Table 4.7. Numbers of genetic markers which showed intrapopulation, interpopulation variation or were invariant in both the KI and GI populations of tammars.

	RFLP*^	Allozyme	Protein	Total
P in KI and GI	10	0	1	11
P in KI	6	3	1	10
P in GI	2	0	1	3
Fixed Difference	15	2	2	19
M in KI and GI	4	23	0	27
Total number of markers tested	22	28	4	

P=Polymorphic; M=Monomorphic; KI=Kangaroo Island; GI=Garden Island
*RFLP=Restriction fragment length polymorphism
^ includes all probe/enzyme combinations
Some markers are included in more than one category if, for example, a marker showed polymorphism and also generated a fixed difference.

Table 4.8. Number of polymorphic (P) and monomorphic (M) restriction fragments identified by each probe/enzyme combination for KI and GI tammars.

	Kangaroo Island										Garden Island									
R E	T		Ba		Pv		E		B		H		Ps		Ms		T		Ba		Pv		E		B		H		Ps		Ms		Fixed
	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	Difference
PROBE																																	
pG115			0	2	0	4	1	6	0	1	0	1							0	3	1	4	0	6	0	1	0	1					Ba,Pv,E
pG1211	0	1	0	2	0	1	0	1	0	2	0	1	2	1			0	1	0	2	0	1	0	1	0	2	0	1	1	2			B
pG862	1	1					0	1					0	2			0	2															T
pB22	0	2	0	1	0	1	1	1	0	3	2	0	0	1			0	2	0	1	0	1	0	2	0	3	2	1	0	1			
pB65	2	2	1	2			0	2	0	1	0	1					0	3	0	2			0	2	0	1	0	1					Ba
pB61	0	1					0	1	0	1	5	0					0	1					0	1	0	1	0	1					
pB54	0	1	0	1	0	1			0	1	0	1					1	1	0	1	0	1			0	1	0	1					
pB3							0	1	0	2	4	0	0	2									0	1	0	2	1	3	0	2			H
pB15	0	1	0	1					0	1	0	1					0	1	0	1					0	1	0	1					B
pB12									2	3	0	1													0	6	0	1					B
pB67	0	4							0	5	1	3					0	3							0	5	0	3					T,B,H
pB72	0	1							0	1							0	1							1	1							T,B
pG1333	2	3							0	6							0	4							0	6							
rRNA					2	3	1	3	2	1	1	2									2	2	1	3	0	2	4	2					Pv,E,B,H
HPRT4.1	0	2			0	1			0	1							0	1			0	1			0	1							
Ex5-13	6	2									0	4					1	4									0	3					H
pG777	3	2			1	4			0	3					0	4	0	4			0	4			0	3					1	2	T,Ms
pG848	0	1			0	1			0	1					0	1	0	1			0	1			0	1					0	1	
Tf34											0	2															0	2					
pG1050	0	2			5	4	1	1	0	1							0	2			3	1	2	1	0	1							Pv,E,B
ME5	12	7			1	9					0	8			0	16	1	13			2	2					2	9			1	15	T,H,Pv,M
DBB	2	8			0	12			6	6	0	7					5	5			0	12			3	6	1	7					T,Pv,B

R E=Restriction Enzyme; T=Taq I; Ba=Bam HI; Pv=Pvu II; E=Eco RI; B=Bgl II; H=Hind III; Ps=Pst I; Ms=Msp I
KI = Kangaroo Island; GI = Garden Island

Table 4.9. Number of polymorphic (P) and monomorphic (M) restriction fragments detected by single locus probes in KI and GI tammars.

	Kangaroo Island										Garden Island									
R E / PROBE	T	Ba	Pv	E	B	H	Ps	Ms	P_e	n_e	T	Ba	Pv	E	B	H	Ps	Ms	P_e	n_e
pG115		M	M	P	M	M			1	5		M	P	M	M	M			1	5
pG1211	M	M	M	M	M	M	P		1	7	M	M	M	M	M	M	P		1	7
pG862	P			M			M		1	3	P								1	1
pB22	M	M	M	P	M	P	M		2	7	M	M	M	M	M	P	M		1	7
pB65	P	P		M	M	M			2	5	M	M		M	M	M			0	5
pB61	M			M	M	P			1	4	M			M	M	M			0	4
pB54		M	M	M	M	M			0	5		M	M	P	M	M			1	5
pB3				M	M	P	M		1	4				M	M	P	M		1	4
pB15	M	M			M	M			0	4	M	M			M	M			0	4
pB12					P	M			1	2					M	M			0	2
pB72	M				M				0	2	P				P				2	2
rRNA			P	P	P	P			4	4			P	P	M	P			3	4
HPRT4.1	M		M		M				0	3	M		M		M				0	3
Ex5-13	P					M			1	2	P					M			1	2
pG848	M		M		M	M		M	0	5	M		M		M	M		M	0	5
P_l	3	1	1	3	2	4	1	0			2	0	2	2	1	3	1	0		
n_l	10	6	7	9	13	12	4	1			10	6	7	8	13	12	3	1		

R E=Restriction Enzyme; T=Taq I; Ba=Bam HI; Pv=Pvu II; E=Eco RI; B=Bgl II; H=Hind III; Ps=Pst I; Ms=Msp I

P_e=total number of enzymes which detect polymorphisms per locus; n_e=total number of enzymes tested per locus; P_l=total number of polymorphic loci detected per enzyme; n_l=total number of loci tested per enzyme.

KI = Kangaroo Island; GI = Garden Island

Table 4.10. Average Percent Difference for all probe/enzyme combinations for KI and GI tammars.

	Kangaroo Island									Garden Island								
R E	**T**	**Ba**	**Pv**	**E**	**B**	**H**	**P**	**M**	**MAPD**	**T**	**Ba**	**Pv**	**E**	**B**	**H**	**P**	**M**	**MAPD**
PROBE																		
pG115		0	0	3.2	0	0			0.64		0	6.0	0	0	0			1.20
pG1211	0	0	0	0	0	0	24.7		3.53	0	0	0	0	0	0	12.0		1.71
pG862	6.2			0			0		2.07	0								0.00
pB22	0	0	0	6.6	0	27.2	0		4.83	0	0	0	0	0	20.0	0		2.86
pB65	4.8	12.0		0	0	0			3.36	0	0		0	0	0			0.00
pB61	0			0	0	41.9			10.47	0			0	0	0			0.00
pB54		0	0	0	0	0			0.00		0	0	13.2	0	0			2.64
pB3				0	0	22.5	0		5.62				0	0	8.4	0		2.10
pB15	0	0			0	0			0.00	0	0			0	0			0.00
pB12					19.0	0			9.50					0	0			0.00
pB67	0				0	5.6			1.87	0				0	0			0.00
pB72	0				0				0.00	37.9				15.8				26.85
pG1333	11.6				0				5.80	0				0				0.00
rRNA			7.9	3.3	11.9	13.3			9.10			8.0	5.3	0	20.1			8.35
HPRT4.1	0		0		0				0.00	0		0		0				0.00
Ex5-13	36.7					0			18.35	11.0					0			5.00
pG777	12.2		5.6		0			0	4.45	0		0		0			13.3	3.32
pG848	0		0		0	0		0	0.00	0		0		0	0		0	0.00
Tf34						0			0.00						0			0.00
pG1050	0		23.5	6.0	0				7.37	0		30.5	17.9	0				12.10
DBB	7.6		0		4.8	0			3.10	4.8		0		8.3	4.8			4.47
ME5	15.7		3.5			0		0	6.73	1.7		5.2			2.7		1.9	2.87
MAPD for all loci									**4.40**									**3.36**

R E=Restriction Enzyme; T=Taq I; Ba = Bam HI; Pv=Pvu II; E=Eco RI; B=Bgl II; H=Hind III; P=Pst I; M=Msp I
MAPD=Mean Average Percent Difference
KI=Kangaroo Island; GI = Garden Island

Table 4.11. Average heterozygosity for all probe/enzyme combinations for KI and GI tammars.

	Kangaroo Island									Garden Island								
RE	**T**	**Ba**	**Pv**	**E**	**B**	**H**	**P**	**M**	**H_A**	**T**	**Ba**	**Pv**	**E**	**B**	**H**	**P**	**M**	**H_A**
Probe																		
pG115		0	0	0.35	0	0			0.070		0	0.35	0	0	0			0.070
pG1211	0	0	0	0	0.02	0	0.35		0.053	0	0	0	0	0	0	0.20		0.028
pG862	0.06			0			0		0.020	0								0.000
pB22	0	0	0	0.49	0	0.33	0		0.117	0	0	0	0	0	0.49	0		0.070
pB65	0.07	0.47		0	0	0			0.108	0	0		0	0	0			0.000
pB61	0			0	0	0.36			0.090	0			0	0	0			0.000
pB54		0	0	0	0	0			0.000		0	0	0.49	0	0			0.098
pB3				0	0	0.36	0		0.090				0	0	0.35	0		0.087
pB15	0	0			0	0			0.000	0	0			0	0			0.000
pB12					0.28	0			0.140					0	0			0.000
pB67	0				0	0.49			0.163	0				0	0			0.000
pB72	0				0				0.000	0.50				0.48				0.490
pG1333	0.35				0				0.175	0				0				0.000
rRNA			0.32	0.16	0.32	0.49			0.322			0.32	0.49	0	0.40			0.302
HPRT4.1	0		0		0				0.000	0		0		0				0.000
Ex5-13	0.42					0			0.210	0.41					0			0.205
pG777	0.30		0.48		0			0	0.195	0		0		0			0.49	0.122
pG848	0		0		0	0		0	0.000	0		0		0	0		0	0.000
Tf34						0			0.000						0			0.000
pG1050	0		0.40		0.09	0			0.122	0		0.48	0.42	0				0.225
DBB	0.09		0		0.11	0			0.056	0.12		0		0.14	0.04			0.059
ME5	0.25		0.49			0		0	0.185	0.38		0.46			0.17		0.41	0.355
H_A over all loci									**0.0962**									**0.0959**

R E=Restriction Enzyme; T=Taq I; Ba=Bam HI; Pv=Pvu II; E=Eco RI; B=Bgl II; H=Hind III; P=Pst I; M=Msp I

H_A=Average Heterozygosity

KI = Kangaroo Island; GI = Garden Island

Table 4.12. Summary of the level of genetic variability detected in the KI and GI populations of tammars by DNA probes, allozymes and serum proteins.

RFLPs	**Kangaroo Island**	**Garden Island**
Number of polymorphic loci detected	16	12
Proportion of polymorphic loci (%)	24.2	18.3
Mean average percent difference (%)	4.40	3.36
Average heterozygosity	0.0962	0.0959
Number of probe/enzyme combinations typed	65	63
Average number of animals typed for each probe/enzyme combination	14.86	7.78
Allozymes/Proteins		
Number of loci	32	32
Number of polymorphic loci	5	2
Heterozygosity	0.034	0.029
Average number of animals examined	60.6	17.0

KI = Kangaroo Island; GI = Garden Island

Table 4.13. Calculations of predicted level heterozygosity for different values of effective population number (N_e) over increasing numbers of generations (t).

		Effective Population Number (N_e)				
		100	200	300	400	500
	100	0.606	0.778	0.843	0.882	0.905
Number	500	0.082	0.286	0.434	0.535	0.606
of	1000	0.007	0.082	0.182	0.286	0.368
Generations	1500	----	0.023	0.078	0.153	0.223
(t)	2000	----	0.007	0.033	0.082	0.135
	2500	----	0.002	0.014	0.044	0.082
	3000	----	----	0.006	0.023	0.050

CHAPTER 5

LINKAGE MAPPING THROUGH RECOMBINATION IN THE TAMMAR WALLABY

5.1 INTRODUCTION

One of the central aims of this research was to develop an efficient system for generating a map of a marsupial genome through recombination. The uses of such a system are several fold.

Comparisons between maps of different species has increased understanding of mammalian genome evolution. To date, the majority of available maps are for eutherian species - the most comprehensive being for humans (HGM 11 1991) and mice (HGM 11 1991; Copeland and Jenkins 1991; Avner et al., 1988). As marsupials and eutherians diverged at between 80-170 million years ago (Hope et al., 1990; Novacek 1992), comparisons between marsupial and eutherian gene maps will add a new dimension to the understanding of mammalian genome evolution. Marsupials are characterised by much lower chromosome numbers than eutherians; the marsupial range is 2n=14 to 32 with two strong modes of 2n=14 and 16 (Hayman 1990) compared to the eutherian range of 2n=6 to 84 (reviewed in White 1973 p.391). A considerable proportion of marsupial karyotypes appear to be conserved over wide evolutionary distances (Hayman et al., 1987; Hayman 1990) suggesting that the rate of karyotypic evolution in marsupials may be slower than in some eutherian lineages. Comparisons of marsupial and eutherian maps may generally help elucidate the evolution of genetic linkage groups and to identify ancient therian chromosome structure and organisation. More specifically, comparisons between marsupials and eutherians may provide further understanding of gene regulation and expression in mammals.

Identification of genetic linkage groups will allow further comparisons to be made between male and female recombination rates in marsupials. In contrast to

generally higher female than male recombination rates in eutherians (Dunn and Bennett 1967), severely reduced recombination rates in females have been reported in two evolutionary distant marsupial species *Sminthopsis crassicaudata* (Bennett et al., 1986) and *Monodelphis domestica* (van Oorschot et al., 1992). In parallel, females of these species have significantly lower numbers of chiasmata per cell than do males, particularly in interstitial regions (Bennett et al., 1986; Hayman et al., 1988). This dramatic difference in chiasmata frequency is not observed in two other marsupial species *Bettongia penicillata* (Hayman et al., 1990) and *Trichosurus vulpecula* (Hayman and Rodger 1990) suggesting that, since the likely level of recombination can be inferred from chiasmata frequencies, severely reduced female recombination might not be a general characteristic of marsupials.

Both eutherians and marsupials have dosage compensation for the extra X-chromosome in females. In eutherians this inactivation is random but in marsupials it is the paternally derived X which is inactivated. In addition, some paternally derived X-linked alleles in marsupials are expressed in some tissues (reviewed in Cooper et al., 1990). An efficient system of generating a linkage map of the X-chromosome which also enables investigation of expression of parental specific alleles, promises to increase our understanding of the initiation and maintenance of X-chromosome inactivation.

Compared to the wealth of data available for eutherian species, few loci have been mapped in marsupials (Graves et al., 1993) and only five genetic linkage groups have been identified. These are ADA-GPI-PI and PGD-SOD-TRF in the Australian species *S. crassicaudata*, (the fat-tailed dunnart) (Bennett et al., 1986); AK1-PI and C6-C7-GPT in the distantly related American marsupial, *M. domesticus* (the gray short-tailed opossum) (van Oorschot et al., 1992; van Oorschot pers. comm.); and GPI-PI in the tammar (van Oorschot and Cooper 1990).

This paucity of genetic linkage information results largely from the difficulty of identifying a marsupial species which firstly exhibits sufficient genetic variation and secondly produces enough offspring for linkage analysis. Difficulties in obtaining

male and female recombination data for marsupials also largely stem from lack of sufficient detectable genetic variation within study species.

In order to overcome this problem an approach of crossing genetically distinct wallabies and then backcrossing F1 hybrids to the parental strains was attempted. A similar approach has been used for mapping studies in several eutherian species. There are two factors to be taken into account when assessing the potential success of such a system. Firstly, the wider the cross (that is, the greater the genetic distance between the parental strains) the more likely the identification of useful genetic differences. This must be balanced however, by a second consideration that the wider the cross, the greater the likelihood of a barrier to successful reproduction.

Two different crosses were attempted during this Ph.D. in an effort to generate a marsupial linkage map . The first was an interspecies cross between the parma wallaby and the black-striped wallaby. They are each others closest relatives (Richardson and McDermid 1978) and were thought to hybridise in captivity (Close and Lowry 1990). This cross was attempted in both directions. No F1 hybrid animals were born during the time of this Ph.D. (discussed in detail in Chapter 3).

The second cross was between two subspecies of tammar wallabies, one from KI in SA and the other from GI in WA. The subspecies hybridise in captivity (W. Poole pers. comm.; Chapter 3). F1s of both sexes were fertile and could be successfully backcrossed to the parental strains (Chapter 3). Whilst successful matings occurred between KI and GI tammars, there appeared to be the beginnings of a barrier to reproduction between the SA and WA populations (Chapter 3).

The KI and GI populations are sufficiently genetically distinct (van Oorschot and Cooper 1988a; Chapter 4) for efficient identification of allelic differences. An additional, but not trivial, advantage of this system is that the reproduction of female tammars can be manipulated so that each female can, theoretically, produce 4-5 offspring per year (Hinds et al., 1990; Chapter 3 for a detailed discussion).

5.2 MATERIALS AND METHODS

Animal husbandry; DNA extraction methods; allozyme, protein and DNA electrophoresis techniques; Southern hybridisation; details of genetic markers used; and statistical methods are discussed in Section 4.2.

The only addition to the description of these techniques which was used for the work discussed in this chapter was that the tissue used for allozyme typing of 10-14 day old pouch young was tail tip.

5.3 RESULTS

In order to detect polymorphism between the two sub-species, KI, GI and F1 animals were typed for 54 genetic markers (Tables 4.2 and 4.3, Chapter 4). Allozyme and protein data was generated by van Oorschot and Cooper (1988a) and Cooper and Bell (unpublished data). Large gene frequency or fixed differences were observed for nineteen of these markers (Table 4.4, Chapter 4). Segregation ratios for fifteen markers for which family data are available do not deviate from expected values (Table 5.1).

5.3.1 Development of a Linkage Map for the Tammar Wallaby Based on Male Recombination Frequencies.

Male F1 hybrids were backcrossed to KI females to produce 107 test cross progeny. Parents and progeny were typed for thirteen of the above markers. The remaining six markers which showed fixed differences between the KI and GI subspecies were not useful for mapping in this case for the following reasons: i) the serum protein markers Pi and plasma esterase show differing electrophoretic patterns in pouch young compared with adults. As about 80% of the test-cross panel were pouch young aged between 10-14 days, results for these two markers were uninterpretable, ii) the cDNA probe pB67 showed a fixed difference by the presence of a band in KI and an absence of this band in GI. As all the progeny had at least one KI chromosome/pair, this marker was uninformative, iii) pG1050 is a mitochondrial

probe, and iv) RNR and GPD are X-linked. As this cross detects male recombination, these probes were not appropriate.

For each pair of markers fathers were phase known double heterozygotes and mothers double homozygotes, and so the data can be subjected to χ^2 analysis for a departure from a 1:1 ratio of parentals to recombinants (Table 5.2). This analysis revealed that pB72 - LALBA form linkage group II and LLP - LPL - pB65 form linkage group III (linkage group I being the GPI-PI group; van Oorschot and Cooper, 1990). The lod score calculations supporting this conclusion and the male recombination frequencies are given in Table 5.3. The probable gene order of linkage group III is pB65-LLP-LPL (the other possible order being LLP-pB65-LPL) but more data are needed (Table 5.4). Figures 5.1-5.5 show RFLP patterns detected by the cDNA probes for LALBA, LLP LPL, pB72 and pB65.

5.3.2 Female Versus Male Recombination Rates

Matings between female F1s or backcrosses and males from either the KI or GI parental sub-species produced 85 test-cross progeny. Families were typed for markers from linkage groups II and III (LLP, LALBA, pB72 and pB65). At these loci, mothers were phase-known heterozygotes and fathers were homozygotes. Of 28 backcross progeny typed for LALBA and pB72 five were recombinants, giving a female recombination rate of 0.18. This rate does not differ significantly from the male recombination rate of 0.07 (χ^2=2.65, p>0.05). Amongst 46 progeny typed for LLP and pB65, 12 were shown to be recombinants, giving a female recombination rate of 0.26. This rate is significantly higher than the male recombination rate between these loci of 0.07 (χ^2=8.72 , p<0.01) (Table 5.5).

5.3.3 X-Chromosome Mapping

Fixed differences were observed between KI and GI for two X-linked markers, RNR and GPD (Figures 5.6 and 5.7 respectively). Sixty nine progeny resulting from crosses between F1 or backcross females who were double

heterozygotes, and homozygous KI or GI males were typed for these two markers. No recombinants were detected.

5.4 DISCUSSION

The potential power of wide crosses, between species or widely separated sub-species, has been exploited most fully in the *Mus. musculus/Mus. spretus* system (Avner et al., 1988; Copeland and Jenkins 1991). The tammar system described here is the best available for marsupial gene mapping through recombination values. Identification of large gene frequency or fixed differences between KI and GI tammars has become substantially more efficient with the use of DNA probes as opposed to the more traditionally used allozyme and protein markers. Seventy percent of DNA probes detected fixed differences between the two populations compared with 13% of biochemical markers.

Of the thirteen autosomal markers for which family data is available, five belong to linkage groups. As the body of information for additional markers increases, so will the probability of detecting linkage between loci.

The gene for LALBA has been mapped on human, cattle and mouse chromosomes 12, 5 and 15 respectively (Womack 1991). LLP appears to be homologous with β-lactoglobulin (LGB) of cattle and possibly with placental protein 14 (PP14) of humans (Woodlee et al., 1993). LGB is in cattle linkage group 2, which also contains the J blood group (Hines et al., 1969); and has also been shown to belong to cattle syntenic group U16 (Womack et al., 1989). PP14 maps to human 9q34 together with the ABO blood group (Bernheim et al., 1991). LPL maps to human chromosome 8p22 (Sparkes et al., 1987) porcine chromosome 14q12-q14 (Gu et al., 1992) and murine chromosome 8 (Davisson et al., 1990) and has also been placed in bovine syntenic group 18 (Treadgill and Womack 1991). These data suggest that markers found on human chromosomes 8p and 9q may be located on the same chromosome in tammars. Thus some preliminary identification of the

relationships between tammar chromosomal regions and their homologues in four eutherian species will become possible as the tammar map grows.

Sequences of the anonymous tammar cDNA clones used in this thesis have been compared with sequences available in GENEBANK (C. Collet pers. comm.). To date, these clones show no significant homology with reported sequences. This was not surprising, since, for example, Adams et al., (1992) have reported that 83% of 2,375 clones isolated from a human brain cDNA library were not related to any known sequences. Linkage groups containing identified genes are the most immediately useful for developing a Type I map (O'Brien 1991), i.e. a map that contains markers for known genes and so can be compared across wide evolutionary distances. Whilst probes containing known gene sequences are directly comparable, anonymous cDNA probes are probably the next most valuable type of marker. These probes can be mapped in other species using *in situ* hybridisation and genetic linkage approaches, usually across wider genetic distances than for anonymous genomic DNA sequences isolated at random. Additionally, in time, the homologies of these DNA will become known making direct comparisons across species possible. There is even the intriguing possibility of making new gene assignments in man and other eutherians where a highly conserved anonymous tammar cDNA can be used for mapping in these species.

Comparison of male and female recombination rates between the linked genes reported in this chapter contrast with results from previous studies in other marsupial groups. Whilst recombination data have been available for only two marsupial species this additional information regarding the tammar wallaby suggests that sex differences in recombination rates may have altered throughout mammalian evolution. *Sminthopsis crassicaudata* and *M. domestica*, belonging to the families Dasyuridae and Didelphidae respectively, show severely reduced female recombination rates. Australian and North American marsupials are thought to have diverged 70 million years ago (Maxson et al., 1975). The macropodids diverged from dasyurids between 55 and 80 million years ago (Hope et al., 1990) and it has now been shown that a

member of this family, the tammar wallaby, exhibits either no significant sex differences or else higher female than male recombination rates, similar to observations obtained in eutherian mammals (Dunn and Bennett 1967). Females of the species *T. vulpecula*, a member of the phalangerids, have lower numbers of chiasmata than males but the difference is not as great as that observed in *S. crassicaudata* and *M. domestica* (Hayman and Rodger 1990). Further, *B. penicillata*, a potoroid (the other main group of wallabies), shows no significant sex difference in chiasmata frequency or distribution (Hayman et al., 1990). Whilst this evidence is indirect, it is also consistent with suggestions that recombination frequencies have altered throughout mammalian evolution.

The chromosomal locations of the loci that form linkage groups in *S. crassicaudata* or *M. domestica* are unknown, and so thus recombination rates cannot be compared to chiasmata frequency patterns. Studies of chiasmata distribution and frequency in *S. crassicaudata* and *M. domestica* have shown that whilst females have significantly lower numbers of interstitial chiasmata than males have, most chiasmata in females are terminal and they were more frequent than terminal chiasmata in males (Bennett et al., 1986; Hayman et al., 1988). This suggests that recombination rates between terminal loci in the females of these species may be greater than between interstitial loci in females. It also suggests that recombination rates between terminal loci in females could be greater than for the same loci in males. LLP has been localised to the interstitial region of chromosome 3 in the tammar wallaby (Westerman et al., 1991). Hence association can be made between sex differences in recombination rates and a specific chromosomal region. Data derived from the tammar wallaby show that higher female than male recombination rates can occur between interstitial loci in a marsupial.

Interspecific crosses between karyotypically distinct mice have shown increased recombination rates compared with the rates noted in intraspecific crosses. These differences were observed only for the female map since F1 males are infertile (Reeves et al., 1991). Both male and female F1 hybrids and backcrosses between KI

and GI are fertile. The two sub-species are karyotypically indistinguishable (Eldridge 1989; Cooper, unpublished data). Segregation ratios from tammar hybrids for 15 loci do not deviate from expected values. There is thus no indication of unusual gene or chromosome behaviour resulting from the sub-specific nature of the cross.

No recombination (n=69) was detected between two X-linked markers, RNR and GPD. Two interpretations of this result are possible. Either the tammar X-chromosome has restricted recombination, or else the loci are closely linked. Since there is no sex difference or significantly higher recombination rates in female than male tammars, the second interpretation seems most likely. This is supported by a report by Johnston and Sharman (1975). A recombination rate of 0.09% (n=11) was inferred between these two loci in *M. robustus robustus* and *M. r. erubescences* backcrosses.

Studies of X-chromosome inactivation in marsupials and eutherians show that the system is not simple. A number of genes on the human X escape inactivation, whereas no genes have been shown to escape inactivation in the mouse. It must be stressed, however, that relatively few genes have been investigated in mice (reviewed in Ballabio and Willard, 1992). Whilst X-chromosome inactivation is random in somatic cells of mice, paternal X-inactivation occurs in extraembryonic tissues.

The marsupial system is no less complicated. The paternal X-chromosome is inactivated in marsupial somatic cells but the system is "leaky". Data is available on the expression of three X-linked genes, GPD, PGK and GLA. These loci are subject to paternal X-inactivation, however expression of paternally derived GPD and PGK alleles has been detected in some tissues and cultured fibroblasts (reviewed in Cooper et al., 1990).

That the inactivation of paternally derived alleles varies from locus to locus, tissue to tissue and species to species has led to the suggestion of two hypotheses. The first suggests that X-chromosome inactivation is a result of locus-specific regulatory factors which act in a piecemeal fashion (Cooper et al, 1977; VandeBerg et al., 1983). The second is the suggestion that X-inactivation is directed from a

specific region of the X-chromosome. Loci close to this region would undergo complete inactivation, whereas more distant loci would be less stringently inactivated (Graves and Dawson 1988).

Studies in humans and mice have provided strong evidence that initiation of X-inactivation requires a cis-acting locus on the X-chromosome. It is known as the X-inactivation centre (XIC and Xic in humans and mice respectively). Deletion studies have broadly localised the inactivation centre to a region between the loci for androgen receptor and phosphoglycerate kinase-1 (Keer et al., 1990). Recent characterisation of a gene within the borders of the inactivation centre , XIST, which is only active on the inactive X-chromosome, has heightened interest in this region (Brown et al., 1991).

Nothing is known of the XIC in marsupials. The high level of conservation of loci surrounding this region in both eutherians and marsupials (reviewed in Watson et al., 1993) is suggestive that the marsupial XIC, if indeed there is such a locus, may be similarly located.

RNR loci are located near the centromere in tammars on Xp. GPD is near the centromere on Xq (Blair 1993). Whilst RNR loci are not subject to dosage compensation, the paternal GPD locus is inactivated in all tissues studied but not in cultured fibroblasts (Johnston and Sharman 1975). GPD must therefore lie within the border of the X-inactivation system.

More data is needed from additional marsupial species before the evolutionary significance of sex differences in recombination rates in mammals can be understood. Difficulties in obtaining data for female chiasmata distribution and frequency in marsupials are compounded by a large number of species being monovular. In addition, lack of genetic variation in study species has lead to difficulties in identifying linkage groups in order to compare male and female recombination rates. These difficulties have also hindered linkage mapping of the X-chromosome. Crosses between sub-species of tammar wallabies allows for efficient identification of linkage groups and provides a system where sex differences in recombination rates can be

compared across an entire marsupial genome. Additionally, this system provides a powerful and unique opportunity to investigate the differential expression of X-linked loci in a marsupial. Not only will this system facilitate far greater comparisons of mammalian genome evolution but will also contribute to the elucidation of one of the more intriguing questions of mammalian genetics - the underlying mechanisms of X-chromosome inactivation.

Table 5.1. Segregation ratios based on family data from test-cross progeny of tammars.

Marker	Number of progeny	χ_1^2
MPI	85	0.01
LLP	80	0.45
PGK-9	96	0.00
CASA	66	3.88
TF	98	0.04
NP	77	3.75
LALBA	85	1.99
pB65	84	0.76
DBB	103	0.48
pB15	104	0.00
LLP	100	0.64
pB12	89	0.10
pB72	56	2.57
RNR	69	0.13
GPD	69	0.13

Table 5.2. χ^2_1 (upper triangle) values for a deviation form a 1:1 ratio of parentals to recombinants based on male recombination rates between autosomal loci typed in test-cross progeny of tammars. The lower triangle is the total number of progeny that were informative at both loci. Numbers in bold type indicate loci which are linked.

	MPI	LLP	PGK-9	NP	CASA	TF	pB65	DBB	pB15	pB12	LALBA	LPL
LLP	0.00 72											
PGK-9	0.69 71	0.29 85										
NP	2.04 83	0.12 75	0.05 74									
CASA	0.43 50	2.64 53	2.29 62	4.17 52								
TF	0.43 84	0.76 84	0.05 82	0.29 87	2.55 57							
pB65	0.06 62	**55.3** **74**	0.11 79	1.00 64	2.09 55	0.12 73						
DBB	1.17 69	8.40 87	2.28 86	4.50 72	1.41 58	0.05 80	0.21 76					
pB15	0.69 71	0.05 78	0.56 87	0.05 74	2.21 64	0.44 82	0.32 77	0.71 90				
pB12	2.60 65	0.01 75	0.80 80	0.13 67	4.44 55	0.63 77	0.01 75	0.30 83	0.19 86			
LALBA	0.64 56	0.89 72	0.01 75	0.83 59	4.96 52	1.80 67	0.00 72	0.22 74	0.32 77	0.01 69		
LPL	0.05 76	**14.4** **80**	0.10 93	2.80 79	5.17 65	0.55 89	**12.5** **82**	4.76 84	0.01 95	7.00 89	1.80 80	
pB72	1.19 41	0.51 49	1.23 52	3.43 42	4.67 32	0.33 48	0.33 48	3.31 51	0.92 53	1.23 52	**34.8** **46**	3.81 59

Table 5.3. Linkage data from test-cross progeny of tammars.

LINKAGE GROUPS	LINKED LOCI	θ_m	SE(θ_m)	cM	n	χ^2_1	Z
II	LALBA-pB72	0.06	0.035	6	46	34.8	9.03
	pB65-LLP	0.07	0.030	7	74	55.3	14.33
III	LLP-LPL	0.28	0.051	32	80	14.4	3.24
	pB65-LPL	0.30	0.050	35	82	12.5	2.78

θ_m = male recombination frequency

SE(θ_m) = standard error of θ_m

cM = map distance in centiMorgans as calculated by Kosambi's formula (1944)

n = total number of informative progeny at both loci

χ^2_1 = for a deviation from a 1:1 ratio of parentals to recombinants

Z = lod score

Table 5.4. Haplotype data of backcross tammars for linkage group III

LPL	LLP	pB65	N	
K	K	K	25	47
G	G	G	22	
G	K	K	13	20
K	G	G	7	
K	G	K	1	2
G	K	G	1	
K	K	G	0	2
G	G	K	2	

LPL = lipoprotein lipase
LLP = late lactation protein
K = Kangaroo Island RFLP inheritance pattern
G = Garden Island RFLP inheritance pattern
n = total number

Table 5.5. Comparison of male and female recombination rates for the linkage groups LLP - pB65 and LALBA - pB72 in the tammar wallaby.

Linkage group	Sex of double heterozygote	n	θ	cM	χ_1^2 (male v female)
LLP- pB65	male	74	0.07	7	
					8.72
	female	48	0.26	31	
LALBA-pB72	male	46	0.06	6	
					2.65
	female	28	0.18	18	

n = total number of backcross progeny typed, θ= recombination rate, cM= map distance in centiMorgans as calculated by Kosambi's (1944) formula, χ_1^2 = for a deviation from a ratio of 1:1 parentals to recombinants, LLP= late lactation protein, LALBA= α-lactalbumin, pB65 and pB72= anonymous cDNA markers.

Figure 5.1. Southern blot analysis of KI, F1 and BX tammar DNA after digestion with Bgl II and subsequent hybridisation with LALBA.

* GI specific fragment
** KI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid; BX = backcross
LALBA = α-lactalbumin

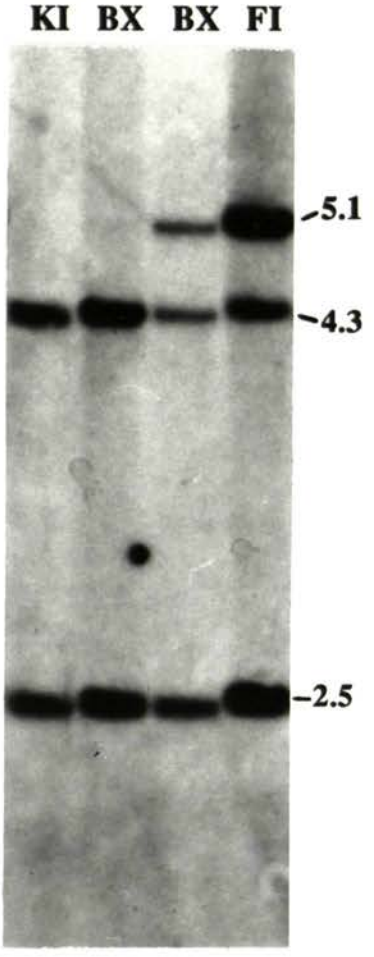

KI
BX
BX
FI
5.1
4.3
2.5

Figure 5.2. Southern blot analysis of KI, F1 and GI tammar DNA after digestion with Taq I and subsequent hybridisation with LLP.

* GI specific fragment
** KI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid
LLP = late lactation protein

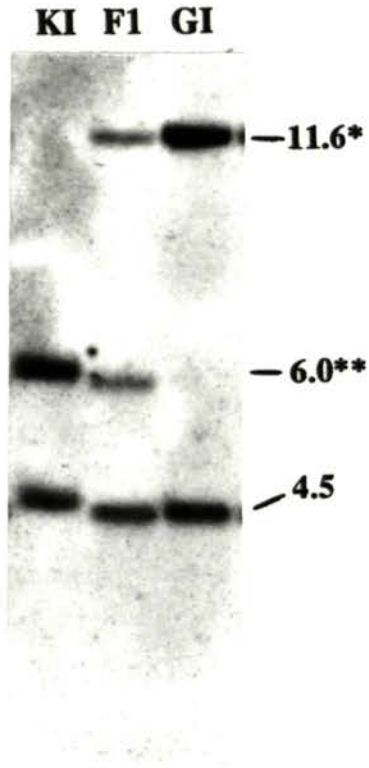

KI
F1
GI
11.6*
6.0**
4.5

Figure 5.3. Southern blot analysis of KI and GI tammar DNA after digestion with Hind III and subsequent hybridisation with LPL.

* KI specific fragment
** GI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island
LPL = lipoprotein lipase

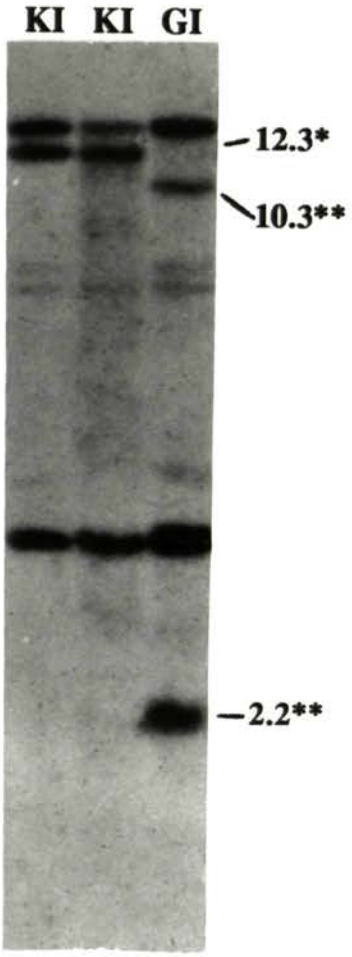

KI
KI
GI
12.3*
10.3**
2.2**

Figure 5.4. Southern blot analysis of KI, F1 and GI tammar DNA after digestion with Taq I and subsequent hybridisation with pB65.

* GI specific fragment
** KI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid
pB65 = anonymous tammar cDNA clone

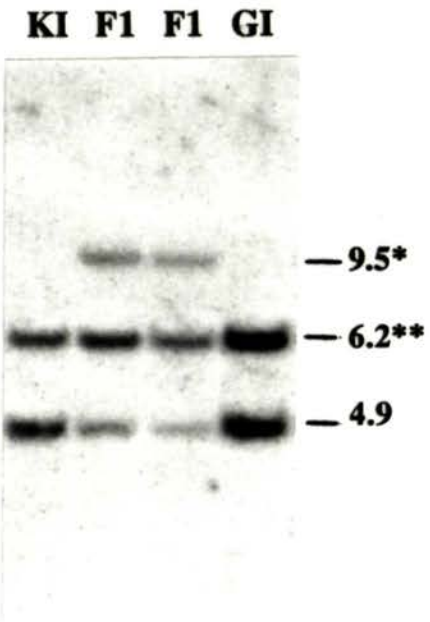

KI
F1
F1
GI
9.5*
6.2**
4.9

Figure 5.5. Southern blot analysis of KI, F1 and GI tammar DNA after digestion with Bgl II and subsequent hybridisation with pB72.

* GI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid
pB72 = anonymous tammar cDNA clone

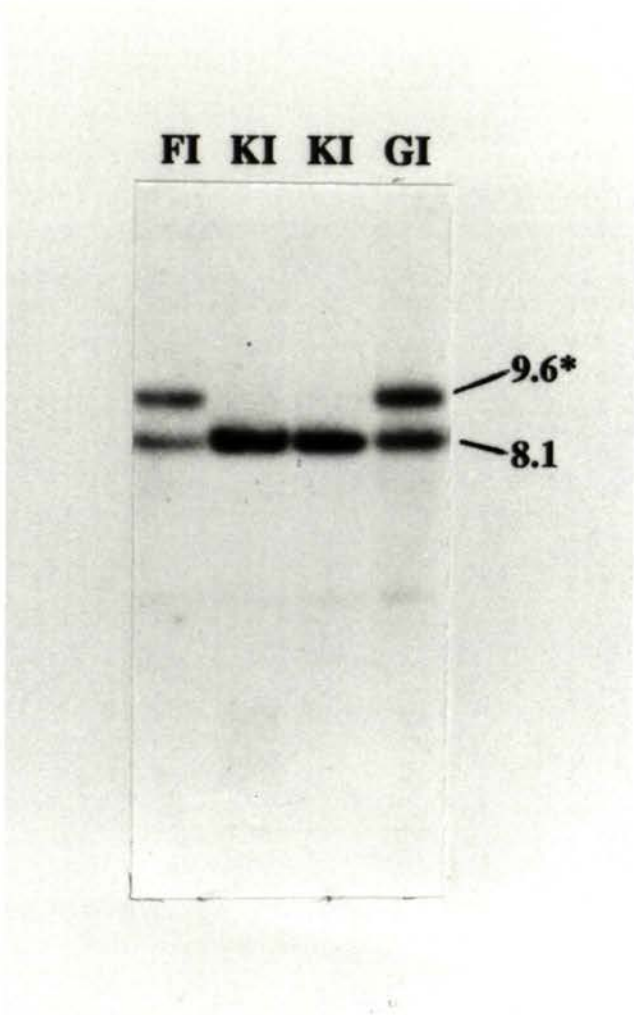

FI
KI
KI
GI
9.6*
8.1

Figure 5.6. Southern blot analysis of KI, F1 and GI tammar DNA after digestion with Bgl II and subsequent hybridisation with RNR.

* GI specific fragment
** KI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid
RNR = ribosomal RNA

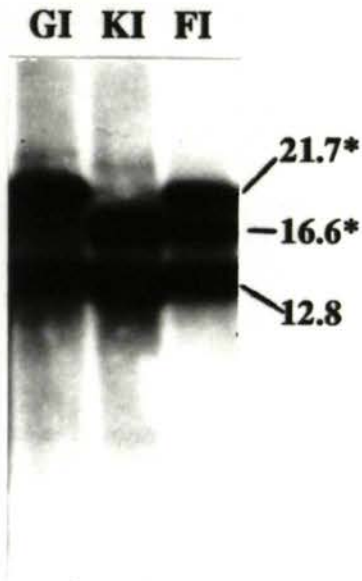
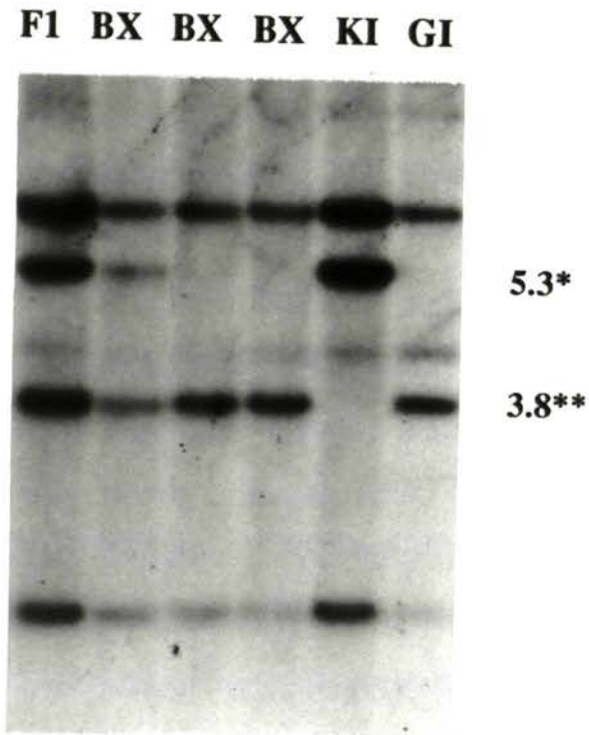

GI KI FI
21.7*
16.6*
12.8

Figure 5.7. Southern blot analysis of KI, F1, BX and GI tammar DNA after digestion with Hind III and subsequent hybridisation with GPD.

* KI specific fragment
** GI specific fragment
Fragment sizes are in kilobases

KI = Kangaroo Island ; GI = Garden Island; F1 = hybrid; BX = backcross
GPD = glucose-6-phosphate dehydrogenase


F1
BX
BX
BX
KI
GI
5.3*
3.8**

CHAPTER 6

LEVELS OF GENETIC VARIABILITY AT MHC CLASS II LOCI IN THE TAMMAR WALLABY

6.1 INTRODUCTION

The major histocompatibility complex (MHC) is a group of closely linked loci which code for cell-surface glycoproteins that mediate cellular immune responses. Class I genes code for the major transplantation antigens that function in graft rejection and the recognition of foreign or infected cells. Class II genes encode alpha and beta chains of heterodimers that are primarily expressed on B cells and macrophages. These antigens are involved in assisting certain T cell subsets in antigen recognition. Class I and Class II β loci have been shown to exhibit extensive levels of genetic polymorphism in the majority of eutherian species studied to date; for example cats (Yuhki and O'Brien 1988; Neefjes et al., 1986): sheep (Chardon et al., 1985a): pigs (Chardon et al., 1985b): mole rats (Nizetic et al., 1985): rats (Palmer et al., 1983) in addition to the much studied mice and human systems. Class II variation has been defined in several species; for example dogs (Sarmiento and Storb 1988): rabbits (Marche et al., 1989): chickens (Andersson et al., 1987): rats (Watters et al., 1987): miniature swine (Pratt et al., 1990): sheep (Scott et al., 1987): cattle (Andersson and Rask 1988), but no quantitative comparative study of the level of polymorphism has been published.

The most striking difference between the divergent marsupial and eutherian mammals is in their mode of reproduction. Eutherian pregnancies are of a relatively long duration and involve intimate connections between fetal placental and maternal uterine tissue. Pregnancy in marsupials is short, young are born in an immature state and the majority of their development occurs in the pouch. With the exception of bandicoots (*Peramelidae*), nothing approaching the invasive placenta of most eutherians is found in

marsupials (Hughes 1974; Padykula and Taylor 1986). It is worth noting, none the less, that all marsupials have a placenta, which makes the use of the term "placental" to differentiate them from eutherians inappropriate.

There is little information on marsupial MHC. Class I MHC responses have been identified in *Monodelphis domestica* (the gray short-tailed opossum) (Infante et al., 1991; Stone et al., 1987), *Setonix brachyurus* (the quokka) (Yadav et al., 1974), *Didelphis virginiana* (the Virginian opossum) (Rowlands 1976) and the tammar wallaby (van Oorschot and Cooper, unpublished data). While characterisation of MHC class I antigens and genes in these species is very limited, the frequency with which transplantation responses and leucocyte antibodies are generated after immunisations suggests that the level of variability for class I genes is comparable with that in eutherian species. In contrast, weak or non-existent mixed lymphocyte culture (MLC) responses have been reported in outbred populations of several species; *M. domestica* (Infante et al., 1991; Fox et al., 1976), *Phascolarctos cinereus* (the koala) (Wilkonson et al., 1992) and *D. virginiana* (Rowlands 1976). Since MLC responses are the result of polymorphism at Class II loci, these data suggest that marsupials, unlike eutherians, have a Class II system which shows limited variation. This question was investigated using the tammar wallaby in a continuance of this species development as a model or reference species for marsupials. This chapter presents data on the level of polymorphism detectable at the molecular level of Class II β- chain-encoding loci of the tammar wallaby, and discusses the relationship between MHC variability and mode of reproduction in both eutherian and marsupial mammals.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Samples were obtained from a fully pedigreed colony of tammar wallabies kept at Macquarie University. This colony, described in Chapter 3, includes outbred animals

from two distantly related sub-species, one from GI in WA and the other from KI in SA. F1 hybrids between sub-species and backcrosses to the KI parent were also used. Data regarding the genetic distance based upon allozymes and serum proteins between these two sub-species is described in Chapter 3.

6.2.2 DNA Extraction, Restriction Enzyme Digestion and Southern Hybridisation

DNA was extracted from both blood and tissue according to Sambrook et al.,(1989) except that tissue samples were initially homogenised and then incubated in 1ml of 50mM Tris pH8.0; 2mM CaCl2; 200µg Proteinase K for 60 mins at 65°C. For Southern analysis, 7ug of DNA was digested with the appropriate restriction enzyme (Progen or Boehringher Mannheim) and electrophoresed on 0.8% agarose gels. DNA was transferred to Hybond-N+ membranes (Amersham) according to manufacturer's instuctions and hybridised with a random-primed ^{32}P dATP labelled probe.

The probe used was Maru-DBB provided by S. Schneider and J. Klein. Maru-DBB contains the complete coding sequence of *Macropus rufogriseus* (the red-necked wallaby) MHC Class II B-chain-encoding gene (Schneider et al., 1991), (Chapter 4).

6.2.3 Statistics

Estimates were made for three values, average percent difference (APD), mean average percent difference (MAPD) and average heterozygosity (H) based on the results of Southern hybridisation with the Maru DBB probe. These values were calculated according to Yuhki and O'Brien (1990); Gilbert et al., (1990) and Stephens et al., (1992).

6.3 RESULTS

DNA from KI and GI tammars was digested with four restriction enzymes and hybridised with the Maru-DBB probe (Figure 6.1-6.4). Data on the fragments found, and the APD, MAPD and H values are given in Table 6.1. For the KI population eight to ten Taq I bands, nine to eleven Bgl II bands, twelve Pvu II bands and seven Hind III

bands were detected. Of these, two Taq I bands and five Bgl II bands were polymorphic. Four of these Bgl II fragments were polymorphic in less than 4% of the cohort. There were no polymorphic bands detected by either Pvu II or Hind III. The APD amongst KI tammars was 7.6% for Taq I, 4.8% for Bgl II and 0% for both Pvu II and Hind III, giving an MAPD of 3.1%. The H for KI tammar MHC class II loci was calculated to be 0.056. For the GI population six to ten Taq I bands, seven to eight Hind III bands, six to nine Bgl II bands and twelve Pvu II bands were detected. Of these, five Taq I bands, three Bgl II bands and one Hind III band were polymorphic. No polymorphism was detected by Pvu II digests. The APD was calculated to be 4.8%, 8.3%, 4.8% and 0.0% for Taq I, Bgl II, Hind III and Pvu II respectively, giving an MAPD of 4.5%. The H value for GI tammar MHC class II loci was calculated to be 0.059.

It has been shown that a lower limit for the number of MHC loci in a species can be calculated from the number of restriction fragments present (Nizetic et al., 1985; Yuhki and O'Brien 1988). From six to twelve fragments were detected by Southern analysis after digestion of tammar DNA with four restriction enzymes, suggesting that there are at least 12 Class II B loci in tammars. This is consistent with the estimates made by Schnieder et al., (1991) that there are approximately 11 Class II β-chain-encoding loci closely related in the red-necked wallaby.

DNA samples from F1 hybrid tammars (KI × GI) (Figures 6.1-6.4) and backcross progeny (F1 × KI) were typed using the Maru-DBB probe. The resulting family analysis showed that RFLP markers followed Mendelian patterns of inheritance (Chapter 4).

6.4 DISCUSSION

The unexpected finding of a weak or non-existent MLC response in marsupials has led to the speculation that marsupials may have significantly reduced MHC Class II

variation in comparison to eutherians (Fox et al., 1976; Infante et al., 1991; Wilkonson et al., 1992; Rowlands 1976). Other possible explanations require mention. Buddle et al., (1992) have shown that the stress of first capture can lead to depressed lymphocyte responses in *Trichosurus vulpecula* (the Australian brushtail possum). Possums held in captivity for longer periods showed normal lymphocyte responses. As the animals used in the studies of marsupial MLC reactivity were adults born and raised in captivity, it seems unlikely that the lack of MLC response is due to stress of capture. It was initially proposed that domestic cats expressed unusually low levels of Class II variation based on weak MLC responses (Pollack et al., 1982). However, with the refinment of culture techniques subsequent studies have shown that domestic cats do indeed exhibit strong MLC response (Wolfe et al., 1984). Fox et al., (1976) argue that the various experimental conditions used in their study of MLC responses in *D. virginiana* rule out the possibility that culture conditions are responsible for the lack of detectable MLC activity in this species.

In this investigation Southern analysis of DNA from two sub-species of tammar wallabies has revealed that these marsupials exhibit a level of class II β polymorphism which appears to be significantly reduced compared to most eutherians. The H value for class II β loci in KI and GI tammars was calculated to be 0.056 and 0.059 respectivily. The MAPD value was 3.1% and 4.5% for KI and GI tammars respectively. Data presented in Stephens et al., (1992) details the MAPD and H values for a number of eutherian species using MHC probes. Using this data set, the tammar wallaby falls between the Southern African cheetah and lions from the Ngorongoro crater. It has been proposed that these feline species have experienced a drastic reduction in population size resulting in a "bottleneck", which was followed by inbreeding (O'Brien and Evermann 1988). Both of these species show exceptionally low levels of MHC variation in comparison with other eutherians (O'Brien et al., 1985; Packer et al., 1991). These data suggest that the tammar wallaby also exhibits significantly reduced levels of

Class II polymorphism.

Since both sub-species of tammars used in this study are from islands, the possibilty that low levels of MHC class II variation is a reflection of an overall decrease in variability due to a population bottleneck must be considered. Data presented in Chapter 4 does not support this notion. The level of heterozygosity in tammars from both KI and GI does not differ significantly from that observed in sheep and humans. Additionally, data presented in Chapter 4 shows that the average heterozygosity of class II loci is approximaltely 50% less than the average heterozygosity in KI and GI tammars.

The role, if any, of the MHC in marsupial reproduction has been the subject of a small number of investigations. Hughes (1974) classified the trophoblast of the tammar yolk sac as being slightly invasive i.e. having rudimentary implantation. Several writers have suggested that marsupials have a short gestation period because, unlike eutherians, they have not evolved a trophoblast capable of successfully resisting an immunological attack upon the embryo by the mother. In particular, Moors (1974) postulated that an allograft reaction was initiated by the presence of the embryo in the uterus, and that this led to birth. Tyndale-Biscoe (1973) and Lillegraven (1975, 1979) have also raised the possibility that the length of marsupial gestation might be limited immunologically, i.e. that birth might be initated by an immunological response of mother to fetus. These speculations are not supported by the little data subsequently collected. Walker and Tyndale-Biscoe (1978) and Rodger et al., (1985) showed that grafting of the sire's skin onto mothers did not impair reproduction, while van Oorschot and Cooper (1988b) could not detect any complement-dependent cytotoxic antibodies to paternally derived antigens in multiparous tammars.

The idea that there should be a relationship between reproduction in eutherians and level of MHC polymorphism goes back to Medaware's influencial 1953 paper on the "immunological paradox of viviparity", from which emerged the term "fetal allograft". Partly through the stimulus of this idea and intense medical interest in the causes of

reproductive difficulties in humans, a large amount of data has accumulated in the intervening years (reviewed in Chaouat 1993). However no clear principals have emerged. Several papers address the question of maintenance of high levels of MHC variability in direct population genetics terms (Hughes and Nei 1988; Potts and Wakeland 1990). Mice preferentially mate with individuals dissimilar to themselves at the MHC (Egid and Brown 1989; Potts et al., 1991; Yamazaki et al., 1976), but form communal nests on the basis of MHC similarity (Manning et al., 1992). Potts et al., (1991) have argued that MHC influenced nonrandon mating patterns are the basis for the greater than expected number of heterozygous offspring observed in some populations of mice. Recognition of MHC geneotypes through olfaction has been reported in mice (Yamazaki et al., 1979), rats (Singh et al., 1987) and humans (Gilbert et al., 1986) providing a possible physiological basis for this behaviour. These mechanisms could also apply to marsupial species. However some evidence exists to suggest that sharing of MHC antigens between mother and fetus in humans and mice is disadvantagous and in the former species may lead to spontaneous abortion (Hedrick 1988; Hedrick and Thomson 1988). Theoretical population genetics studies indicate that a selective advantage for maternal-fetal MHC disparity could account for high levels of MHC variability (Hedrick 1988; Hedrick and Thomson 1988).

A more systematic and quantitative evaluation of the level of MHC class I and II variability in both eutherians and marsupials is now desirable. It would also be of especial interest to study bandicoots because of their invasive chorioallantonic placenta, and monotremes because they are egg-laying. As indicated in the introduction, no quantitative comparative study has been published for eutherian class II genes. On the evidence available, it seems that marsupials could have class I variation comparable in extent to that in eutherians, but have a lesser level for class II. If this impression were to be substantiated, the possibility that MHC class II variation is connected to their respective modes of reproduction would have to be seriously considered.

More generally, our lack of a detailed description of the MHC of any marsupial acts as a barrier to a complete understanding of its evolution in mammals. The existence of fully pedigreed colonies of genetically diverse animals such as the tammar colony used here now makes such a description possible.

Table 6.1. Restriction fragment length polymorphisms of class II β-chain-encoding genes of GI and KI tammars.

Restriction Enzyme	**Taq I**		**Pvu II**		**Bgl II**		**Hind III**	
Subspecies	GI	KI	GI	KI	GI	KI	GI	KI
No. animals examined	19	5	5	5	20	47	5	5
	12.0	12.0*	12.8		15.9		14.1	14.1
		10.0	11.2	11.2	11.5*	11.5	12.4	12.4
	9.0*	9.0*	9.6	9.6			11.5	11.5
	7.9	7.9	8.1	8.1	8.0	8.0	10.7	10.7
	6.9*	6.9	7.6	7.6	6.9*	6.9*	9.3*	
	5.3*		6.9	6.9		6.4	7.8	7.8
	4.9*	4.9	6.0	6.0		5.6*	6.8	6.8
	4.2	4.2	5.5	5.5	4.9	4.9	5.5	5.5
	3.7	3.7	4.1	4.1	4.2	4.2		
	3.2*	3.2	2.6	2.6		3.7*		
	1.6	1.6		2.4	3.2	3.2*		
			2.2	2.2	3.1*	3.1*		
			1.7	1.7	2.8	2.8		
Total	6-10	8-10	12	12	6-9	9-12	7-8	7
Polymorphic	5	2	0	0	3	6	1	0
APD^	4.8%	7.6%	0%	0%	8.3%	4.8%	4.8%	0%

* indicates polymorphic fragments

^ APD = Average percent difference (Yuhki and O'Brien 1990)

Figure 6.1. Southern blot analysis of KI, GI and F1 hybrid DNA after digestion with the restriction enzyme Taq I and subsequent hybridisation with Maru-DBB.

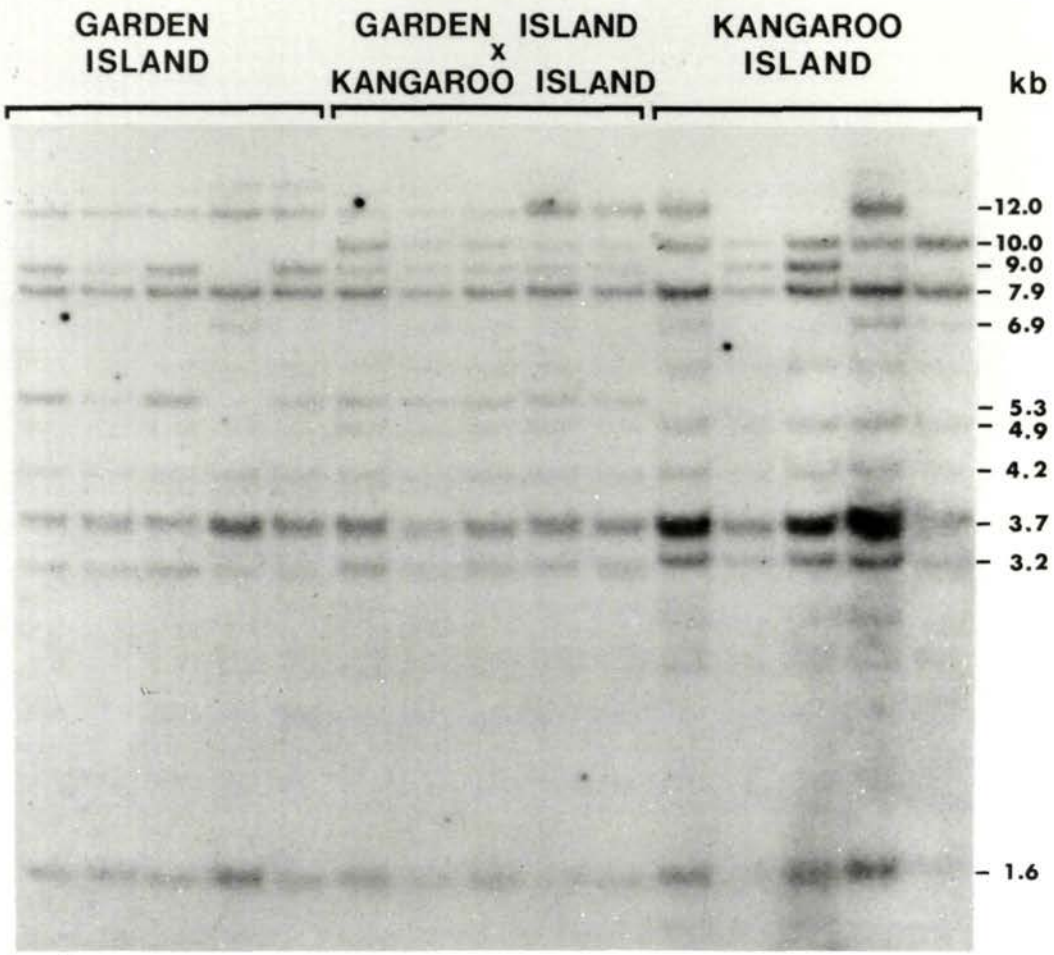

GARDEN ISLAND
GARDEN ISLAND x KANGAROO ISLAND
KANGAROO ISLAND
kb
12.0
10.0
9.0
7.9
6.9
5.3
4.9
4.2
3.7
3.2
1.6

Figure 6.2. Southern blot analysis of KI, GI and F1 hybrid DNA after digestion with the restriction enzyme Bgl II and subsequent hybridisation with Maru-DBB.

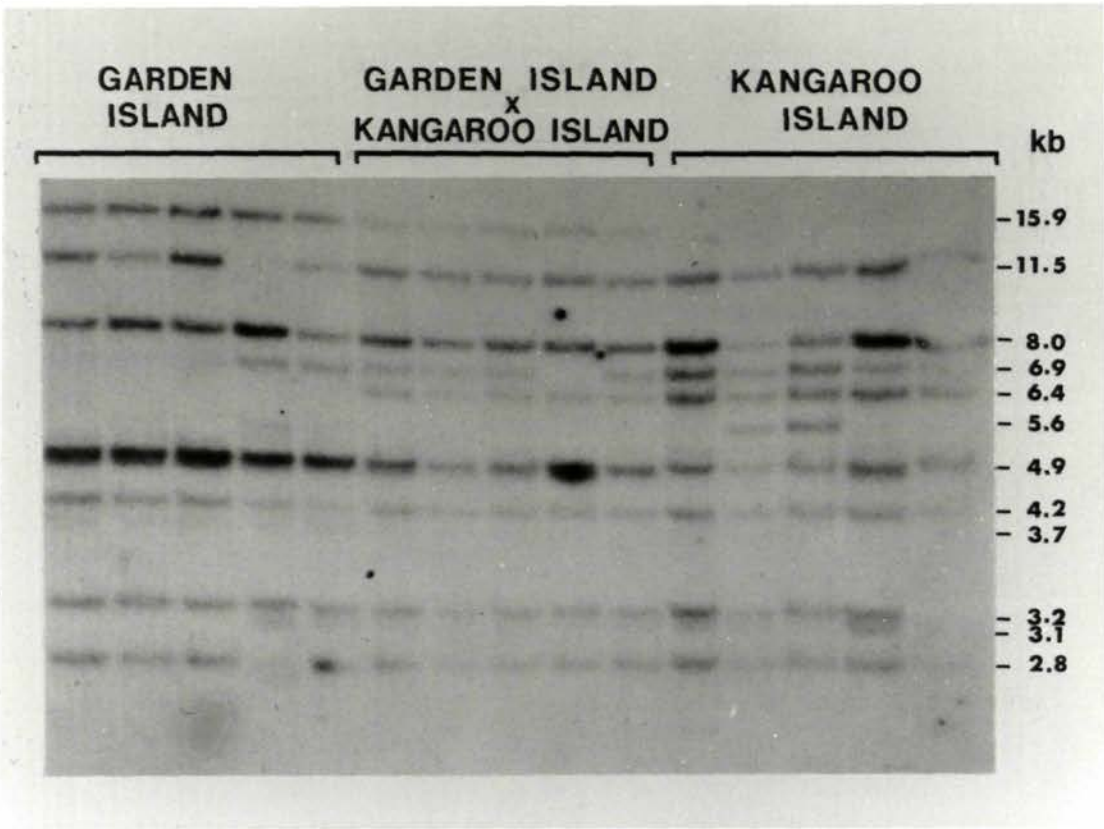

GARDEN ISLAND
GARDEN ISLAND x KANGAROO ISLAND
KANGAROO ISLAND
kb
15.9
11.5
8.0
6.9
6.4
5.6
4.9
4.2
3.7
3.2
3.1
2.8

Figure 6.3. Southern blot analysis of KI, GI and F1 hybrid DNA after digestion with the restriction enzyme Pvu II and subsequent hybridisation with Maru-DBB.

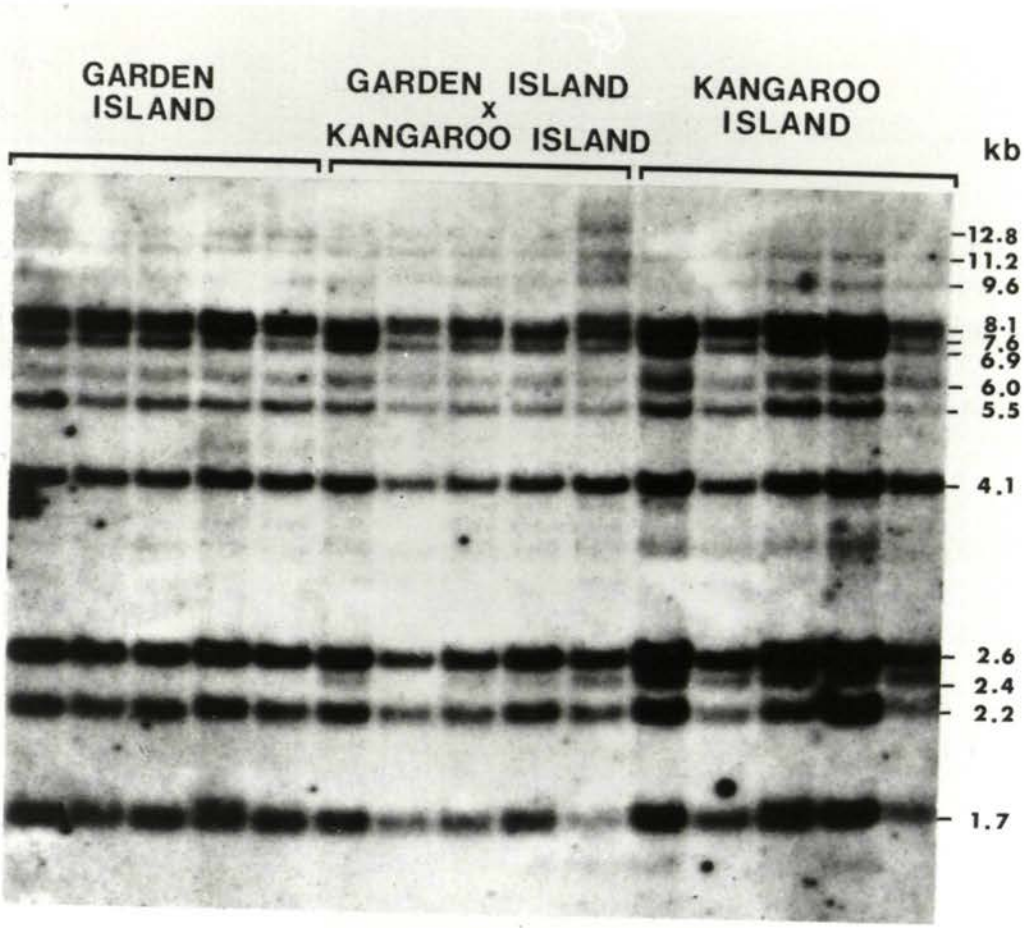
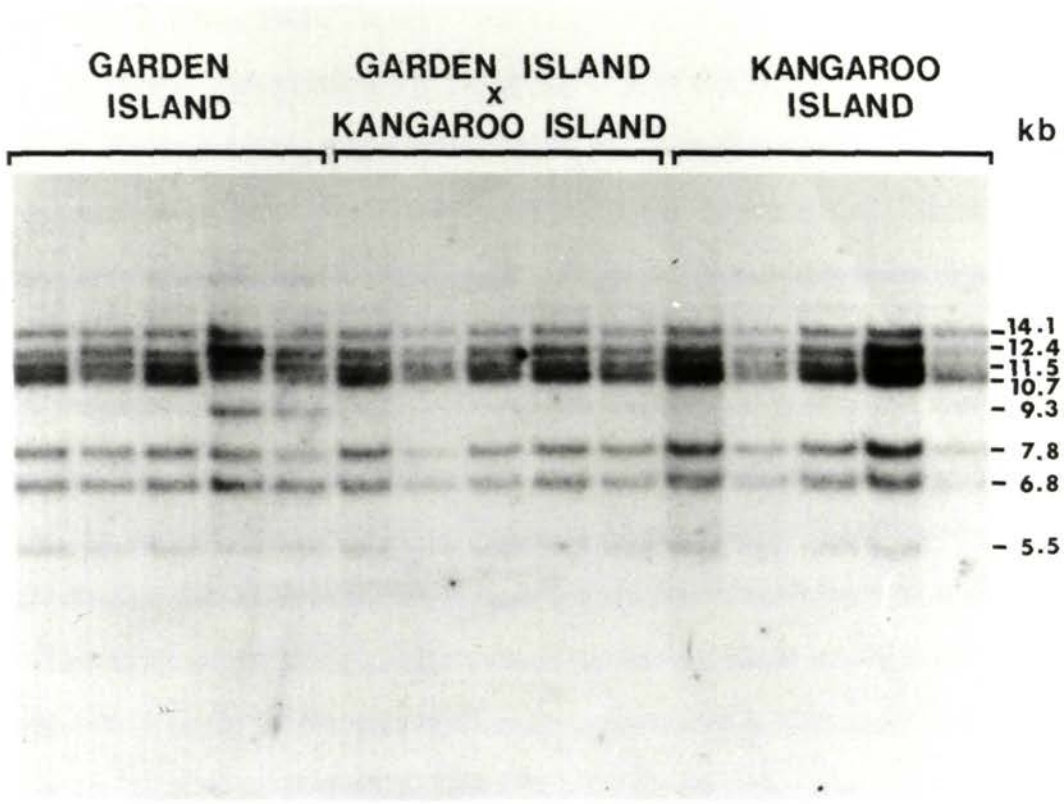

GARDEN ISLAND
GARDEN ISLAND x KANGAROO ISLAND
KANGAROO ISLAND
kb
12.8
11.2
9.6
8.1
7.6
6.9
6.0
5.5
4.1
2.6
2.4
2.2
1.7

Figure 6.4. Southern blot analysis of KI, GI and F1 hybrid DNA after digestion with the restriction enzyme Hind III and subsequent hybridisation with Maru-DBB.


GARDEN ISLAND
GARDEN ISLAND x KANGAROO ISLAND
KANGAROO ISLAND
kb
14.1
12.4
11.5
10.7
9.3
7.8
6.8
5.5

CHAPTER 7

CONSERVATION GENETICS OF THE PARMA WALLABY

7.1 INTRODUCTION

As has been stated, one of the central aims of this Ph.D. was to develop an efficient system for mapping a marsupial genome through recombination. Two approaches were used initially in attempts to establish such a system. The first of these systems was an intraspecies cross between South Australin and Western Australian tammars which were thought to be genetically distinct. This system has been the subject of discussion in the Chapters 3-5 of this thesis. The second system was to attempt a wider cross, that is an interspecies cross. The parma wallaby and the black-striped wallaby have been identified as each others closesests relatives based on allozyme data (Richardson and McDermid 1978). The two species are thought to hybridise in captivity (Close and Lowry 1990). Attempts to mate parmas and black-striped animals to produce test-cross progeny for mapping studies were unsuccsessful. Hence this particular cross was not suitable for the main purpose of this Ph.D. However, data gathered as a result of the initial interest in these species has provided important information regarding the conservation of the parma wallaby. These data and conclusions drawn from them are reported here.

The conservation of the parma wallaby has been a matter of widespread discussion over the last thirty years (Kirsch 1967; Wodziki and Flux 1967; Frankel and Soule 1981; Strahan 1983; King 1990), because it exemplifies a number of important issues which arise in the conservation and management of Australian marsupials. It is the smallest member of the genus Macropus, weighing between 3 and 5 kg (Warburton and Sadlier 1990). Native only to Australia, it was formerly distributed in regions within the Great Dividing Range of New South Wales (Maynes 1977). Parma wallabies were

thought have become extinct (Ride 1957) until the rediscovery of a large population on Kawau Island, New Zealand in 1967 (Wodzicki and Flux 1967). This population originates from a shipment of Australian wallabies released by Sir George Grey in 1870. Little documentation is available of this event. Founder numbers are unknown, and so it is possible that this colony has a restricted genetic base. Parmas on Kawau Island were used to supply zoos and establish breeding colonies throughout the world. Subsequently, Maynes (1974) described a colony of parmas still existing on the mainland of Australia in the Moonpar State Forest, New South Wales (30°12'S,152°41'E). Further isolated populations have been described in the northern regions of the Great Dividing Range. There have been no reported sightings of parma wallabies in the Illawarra or central coast regions to date (Maynes 1977). Whilst mainland colonies of parmas are locally common, their ranges are very small. Parmas are one of a number of marsupials which are of a size defined as most vulnerable to predation by dingos, feral cats and foxes. This range is called the "critical weight range" (Burbidge and N. McKenzie 1989). As all of these predators occur in the North Coast area (Maynes 1974), parmas must be considered vulnerable, if not endangered (Maynes 1976a). Parmas were deemed protected by the New Zealand government in 1969, but this was revoked in 1984 after the rediscovery of the Australian populations.

The black-striped wallaby is one of the largest wallabies; males weigh up to 20kg (Warburton and Sadlier, 1990). Black-stripes were thought to have been released on Kawau Island by Sir George Grey along with parmas, tammar wallabies, *Macropus rufogriseus rufogriseus* (Bennett's wallabies), *Petrogale penicillata* (the brush-tailed rock-wallaby), and *Wallabia bicolor* (the swamp wallaby). Since black-striped wallabies have disappeared from Kawau Island, there remains the question of whether they did so through introgression into the parma population. Thus it is possible that Kawau Island parmas and their derivatives in zoos and breeding colonies may have genetic material from black-striped wallabies, potentially compromising their suitablity for reintroduction

to Australia. In addition, the history of parmas on Kawau Island suggests the possibility of reduced genetic variablity. This chapter addresses four questions: (i) can parma/black-stripe crosses be used for genetic linkage mapping? ii) what is the level of genetic variability of New Zealand derived parmas? (iii) can parma and black-striped wallabies hybridise readily? and, (iv) if so, has there been any detectable genetic exchange between parma and black-striped wallabies on Kawau Island?

7.2 METHODS

7.2.1 Animals

Colonies of parmas and black-stripes were maintained at Macquarie University. New Zealand derived parma wallabies were obtained from Kawau Island either directly in 1972/3 or indirectly from there via Healesville Sanctuary in Victoria in 1986 and maintained as a non- pedigreed colony. Additional material was also obtained from four New Zealand derived animals from Mr. Peter Pigott's colony, Mt. Wilson, NSW. Two parmas donated to Macquarie University by New South Wales University, said to have originated from the Gosford region were also used in this study. Two black-striped males were obtained from Perth Zoo in 1985. Subsequently, three adult black-striped animals and blood samples from an additional ten animals were donated in 1991 by Mr. Peter Johnson, Queensland National Parks and Wildlife Service, Townsville. One presumptive F1 hybrid (parma x black-striped) was given to Macquarie University by Perth Zoo in 1985 and was mated to parma bucks.

7.2.2 Allozyme Electrophoresis

Allozyme electrophoresis was performed on red blood cell lysates according to Richardson et al., (1986) for nine loci: glucose phosphate isomerase (GPI), enolase (ENOL), asparate aminotransferase (AAT/GOT), lactate dehydrogenase (LDH; 2 loci),

haemoglobin (Hb), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH) and superoxide dismutase (SOD). This work was carried out by Cathy Watson and Jenny Donald.

7.2.3 Isoelectric Focusing

Isoelectric focusing (IEF) for albumin (ALB) was carried out using a modification of the technique described by Righetti (1983). Ampholines of the pH ranges pH=3.5-5, pH=5-7 and pH=7-9 were mixed in equal quantites and used. This work was carried out by Cathy Watson and Gay Lynn Woodlee.

7.2.4 DNA Extraction, Restriction Enyme Digestion and Southern Hybridisation

DNA was extracted from whole blood according to Sambrook et al., (1989). For Southern analysis, 7 µg of DNA was digested with the appropriate restriction enzyme (Progen or Boehringer Mannheim) and electrophoresed on 0.8% agarose gels. DNA was transferred to Hybond- N^+ membranes (Amersham) according to manufacturer's instructions and hybridised with random-primed ^{32}PdATP labelled probes.

The probes used were B10 which contains 1401 bases of anonymous cDNA from *P. assimilis* (the allied rock-wallaby) (Odorico et al., 1992); a PCR amplified product from *M. robustus* (wallaroo) cDNA of exons 5-13 of the glucose-6- phosphate dehydrogenase gene (GPD) (P. Johnston pers. comm.); pG862, pG1211 and pG3 which contain tammar late lactation protien (LLP), α-lactoglobulin (LABLA) and lipoprotein lipase (LPL) sequences respectively (Collet et al., 1989; Collet et al., 1990; Collet and Joseph 1994); pB72, an anonymous tammar cDNA clone (C. Collet pers. comm.); and ME5 which contains the first 630 bases of the phosphoglygerate kinase 1 (PGK 1) from tammar (Zehavi-Feferman and Cooper 1992). There are two functional PGK genes and a number of pseudogenes in macropodids (Cooper et al., 1994), and ME5 behaves as a multilocus probe.

7.2.5 Hybridisation Experiments

Attempts to hybridise parma and black-striped animals began in 1985 with the receipt by Macquarie University of a presumptive F1 hybrid female bred in captivity from Perth Zoo. She was subsequently mated to parma bucks and the resulting progeny were also backcrossed to parma animals.

Two black-striped males were kept with 5-7 parma females for periods up to 12 months in 1985-1986. In 1991, two male and one female black-striped wallabies were received from Townsville. Matings were set up between each black-striped male and three parma females, and between the black-striped female and three parma males. Work carried out before 1991 was supervised by others.

7.2.6 Statistics

Estimates were made for two values, average percent difference (APD) and average heterozygosity (H). These values were calculated according to Yuhki and O'Brien (1990), Gilbert et al., (1990) and Stephens et al., (1992).

7.3 RESULTS

7.3.1 Level of Genetic Variability Within Parmas

Four parmas, two New Zealand derived (NZ) and two Australian (A) animals, were typed for the nine enzyme loci described in the methods using allozyme electrophoresis. In addition, fourteen NZ and two A parmas were typed for ALB. No polymorphism was detected at any of these loci.

DNA from twelve NZ and two A parmas was hybridised with LLP after digestion with Taq I, Bgl II and Pst I. Also DNA from four NZ and two A parmas was digested with Hind III and Taq I and hybridised with LALBA. Taq I digested DNA was also

hybridised with LPL. No polymorphism was detected either within or between the NZ and A populations. An additional four NZ parmas were analysed for a further seventeen restriction enzyme/probe combinations, of which thirteen were monomorphic and four revealed intrapopulation polymorphism (Table 7.1). DNA from fifteen parmas (thirteen NZ and two A) was digested with Taq I and subsequently hybridised with ME5. Eleven constant bands and seven polymorphic bands were detected (Figure 7.1). Twelve unique phenotypes were observed (Table 7.2). Phenotype 1 was shared by three NZ animals and phenotype 2 was shared by two NZ animals. Whilst both A animals had unique phenotypes, they revealed all constant bands and did not reveal any new alleles. The H value and APD, based on the ME5 results, were calculated to be 0.17 and 7.7% respectively.

7.3.2 Comparison Between Parma and Black-Striped Wallabies

Allozyme electrophoresis was performed on three black-striped animals for the nine loci previously described. All animals were monomorphic, showing identical patterns to parma. Eleven black-striped wallabies were typed for albumin revealing a fixed difference to parmas previously described by Richardson and McDermid (1978). Ten of the eleven animals showed the common black-striped wallaby band and one animal was a heterozygote for both the parma and black-striped bands. Based on these data and on allozyme data collected by Richarson and McDermid (1978), the two species have a Nei genetic distance of 0.207 (calculation by DW Cooper based on results in Richardson and McDermid (1978)).

DNA from four black-striped animals was digested with various restriction enzymes and hybridised with LALBA, LPL, GPD, LLP and B10. Fixed differences between parma and black-striped animals were observed with LALBA, LPL, ME5 and B10 with Taq I and GPD with Pst I. Parma and black-striped DNA revealed the same monomorphic fragments for LALBA with Hind III, GPD with Bgl II, B10 with Bgl II and

Pst I, and LLP with Bgl II, Taq I and Pst I (Table 7.3). Intrapopulation polymorphism within black-striped wallabies was only detected with the ME5 probe (Fig 7.1).

7.3.3 Hybridisation of Parma and Black-Striped Wallabies

The presumptive F1 hybrid female from Perth Zoo was mated with parma males and produced two female offspring, both of which were successfully backcrossed again to parma males. Female progeny were continued to be crossed with parma bucks. Thirteen backcross progeny were produced, seven females and four males. No progeny have been produced by two male backcrosses over a three year period of potential matings with 4-7 parma females. Measurements were taken for head, ear, tail, pes, leg and arm length, weight and testes size of putative backcross animals and are presented in Table 7.4. No F1 progeny have resulted from attempts, during the periods 1985-1986 and 1991-1993, to cross male parmas with female black-striped wallabies or male black-striped wallabies with female parmas.

7.3.4 Data from F1 Hybrid and Backcross Individuals

The original animal from Perth Zoo which was reported to be a hybrid (black-stripe x parma), was typed for LALBA, LPL, ALB LLP and ME5. In each case, where a fixed difference was observed between black-stripes and parmas, the animal was heterozygous, supporting its hybrid origins (Figure 7.2). Allozyme electrophoresis for the nine loci described in the methods section was performed on two presumptive backcross animals. Both showed identical monomorphic patterns to those seen in parmas and black-stripes. DNA from four putative backcross animals was digested with Taq I and hybridised with the ME5 probe. One of these animals revealed each parma and black-striped specific fragment. The remaining three animals revealed combinations of some parma and some black-striped specific fragments. In addition, seven animals were typed for ALB, three showed both black-striped and parma bands and the remaining four

revealed only the parma band (Figure 7.2 and data not shown).

7.4 DISCUSSION

It is clear from data presented here that whilst hybridisation between parma and black-striped wallabies is possible it does not occur readily enough for the two species to be used for mapping studies. However, important conservation issues regarding parmas can now be addressed.

Despite the rediscovery of parma wallabies on the mainland of Australia in 1974, the continuance of the species in the wild here cannot be certain. Large sections of suitable habitat have been lost to farming and logging since European settlement and the introduction of effective predators (feral cats and foxes) (Maynes 1977) renders the remaining small and disconnected populations of parmas highly vulnerable. As with many species of Australian native fauna, strategies such as reintroduction programs, are being assessed for their application to the conservation of vulnerable wildlife. Here I have attempted to assess the suitability of New Zealand derived parmas for captive breeding colonies and reintroduction to regions of mainland Australia previously or presently inhabited by the species. Historical accounts suggest that after the introduction of parmas to Kawau Island there was a population explosion followed by a severe population reduction (due to hunting) when "not more than a dozen or two" remained (Wodzicki and Flux 1967). As the size of the Kawau Island founder population is unknown, and a population bottleneck may have occurred subsequently, it was possible that New Zealand parmas had restricted genetic variation making them unsuitable for either program. Genetic variability in New Zealand parmas was assessed by calculating the H and APD values based on Southern hybridisation with the ME5 probe, and was found to be 0.17 and 7.7% respectively. These animals were from a non-pedigreed colony and hence their relatedness is unknown. Despite this, their level of genetic variation does not differ

significantly from similar data obtained for 41 unrelated Kangaroo Island tammar wallabies (Cooper et al., 1994). Analysis of this data shows that H and APD are 0.246 and 15.7% respectively.

Comparison of restriction fragment length polymorphism (RFLP) patterns between NZ and A parmas did not reveal any novel alleles or fixed gene differences between populations, suggesting that significant fixation of alleles has not occurred in the Kawau Island population due to drift or inbreeding.

The additional complication of possible gene flow between black-striped and parma wallabies has also been addressed. The data presented here supports the hybrid origins of the animal received from Perth Zoo. This animal was heterozygous at all loci tested for which fixed differences were observed between black-stripes and parmas. Data from putative backcross animals supports their backcross origins as each individual revealed combinations of parma and black-striped specific fragments. However, results of breeding experiments indicate that there is some barrier to reproduction between the species. No hybrid progeny have been produced between possible matings of 6-7 parma females and two black-striped bucks at Macquarie University over the periods 1985-1986 and 1991-1993. This suggests that while hybridisation is possible, it may occur extremely rarely. Further , two backcross males have failed to reproduce with 6-7 parma females over a three year period despite successful female backcross matings, suggesting that males may be sterile. This is consistent with the conclusions of Haldane (1922) that infertility is more common in male than female hybrid animals. Testes size of two male backcross animals were smaller than most parma and all black-striped bucks further suggesting infertility (Table 7.4). The data on both of these species is very limited however, reflecting the lack of basic biological information available - particularly for the black-striped wallaby. In addition, no black-striped specific RFLP fragments were detected in any parmas tested, arguing strongly against the possibility of black-striped wallabies having introgressed into the parma population to any degree. Hence, it can be

proposed that genetic exchange, if indeed there was any at all, between parma and black-stripes in New Zealand does not jeopardise the genetic integrity of the Kawau Island parma population. In fact, Ride (pers. comm.) has questioned whether Sir George Grey did introduce black-striped wallabies to Kawau Island at all. The identification of black-stripes as being in Kawau (Wodzicki 1950) is doubtful as literature records are unsubstantiated. Skins (without skulls) from animals captured on Kawau Island were identified by Le Souef (1929-1931) as black-striped wallaby. But these skins were subsequently re- examined by Ride in 1958 who considered that they were probably parma; this led to the discovery of the species on Kawau Island (Ride 1970). Secondly, a female with a pouch young (found dead from poisoning) was identified as a black-striped wallaby in 1954 based on weight, head and body measurements (King 1990). Upon review it is clear that these measurements fall outside the range for black-stripes. However, these measurements do fall into the range for *W. bicolor* (swamp wallabies) which are known to occur on Kawau Island.

All these data suggest that New Zealand derived parmas do not have restricted genetic variation and have not hybridised extensively or at all with black-striped wallabies. In these terms they would be deemed suitable for captive breeding colonies and reintroduction to mainland Australia. However, other aspects of New Zealand derived parmas suitability for re- introduction to Australia, such as behavioural adaptations, must be considered. Despite limited data, significant biological differences such as reproductive strategies have been observed between NZ and A parmas (Maynes 1973, 1977). Parma wallabies have no known predators apart from man on Kawau Island and animals held in captive breeding colonies and zoos may have no predators at all. These populations may have altered or lost some predator-prey responses making them highly vulnerable to dingos, feral cats and foxes found in their native habitat. Attempts to reintroduce have failed to date with the most significant loss of animals being to fox, cat or dog predation (Short et al., 1992). Adaptive behavioural responses could be equally as important as

genetic variability in assessing the success of any reintroduction program. Survival of the species in Australia could ultimately rely on efforts being directed towards maintenance of remaining suitable habitat and the development of methods to eradicate exotic predators. The importance of Kawau Island as a refuge for parma wallabies should not be underestimated.

Table 7.1. RFLP data obtained for four NZ or * twelve NZ and two A or ** two NZ and two A parma wallabies.

	Bgl II	Pvu II	Pst I	Taq I	Hind III
pB72	P	M	M	M	P
LPL	P	M	M	M**	M
LALBA	M	M	P	M**	M**
B10	M		M	M	
GPD	M		M		
LLP	M*		M*	M*	

M = monomorphic pattern
P = polymorphic pattern
NZ = New Zealand
A = Australian mainland

Table 7.2. Presence/absence of Taq I fragments detected by ME5 in 15 parma wallabies

Phenotype number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	No. of animals
1*	+	+	--	+	+	--	+	+	+	--	+	+	+	+	+	+	+	+	1
2*	+	+	+	+	+	--	+	+	+	--	--	+	--	+	+	+	+	+	1
3	+	+	+	+	+	+	+	+	+	+	+	+	--	+	+	+	+	+	3
4	+	+	+	+	--	+	+	+	+	+	+	+	--	+	+	+	+	+	2
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
6	+	+	+	+	+	--	+	+	+	+	+	+	+	+	+	+	+	+	1
7	+	+	--	+	+	+	+	+	+	+	+	+	--	+	+	+	+	+	1
8	+	+	--	+	--	+	+	+	--	+	+	+	--	+	+	+	+	+	1
9	+	+	+	+	+	+	+	+	+	+	--	+	--	+	+	+	+	+	1
10	+	+	+	+	+	--	+	+	+	+	+	+	--	+	+	+	+	+	1
11	+	+	+	+	+	+	+	+	--	--	+	+	--	+	+	+	+	+	1
12	+	+	+	+	+	+	+	+	+	+	+	+	--	+	+	+	+	+	1

* indicates Australian parma wallabies

Table 7.3. Comparison between parma and black-striped RFLP patterns generated by various probe/ enzyme combinations.

Probe	Enzyme	Parma	Black-striped
LABLA	Taq I	2	1
	Hind III	1	1
LPL	Taq I	1,4	2,4 or 2,3
B10	Taq I	2,3	1,3
	Bgl II	1,2	1,2
	Pst I	1,2	1,2
GPD	Pst I	1,2	2
	Bgl II	1	1
LLP	Bgl II	1	1
	Taq I	1	1
	Pst I	1	1

Based on two Australian mainland, two New Zealand parma wallabies and four black-striped wallabies.

Table 7.4. Comparison of body measurements of four putative backcross animals with parma and black-striped males.

	Sex	Weight (kg)	Ear (mm)	Head (mm)	Pes (mm)	Arm (mm)	Testes length (mm)	Tail (cm)	Leg (cm)
D	M	11.6	71.1	230.0	255.0		39.8	69.4	34.5
D	M	12.1	73.1	281.0	260.0	245.0	35.4	65.6	36.5
D	F	6.6	78.2	205.0	250.0			58.1	32.5
DP	M	6.5	70.0	122.7	168.6	114.0	26.6	55.5	31.5
DP	M	8.5	81.0	130.0	168.7	168.7	28.1	53.5	34
DP	F	5.2	84.0	124.2	142.0	120.0		46.7	28.4
DP	F	5.7	83.9	131.4	152.4	140.4		52.5	29.8
P[a]	M	4.9	68.4	113.0		103.3	27-33	50.2	21.2
P[a]	F	3.97	66.8	109.4		90.2		47.5	20.4

DP = putative backcross animals
P = parma
D = black-striped
[a] = data taken from Maynes, 1976b and Maynes, 1977. All other data are from individual animals held at Macquarie University.

Figure 7.1. Hybridisation of the ME5 probe to parma and black-striped DNA after digestion with TaqI.

* indicates polymorphic fragments based on 6 black-striped and 15 parma wallabies. Fragment sizes are in kilobases.

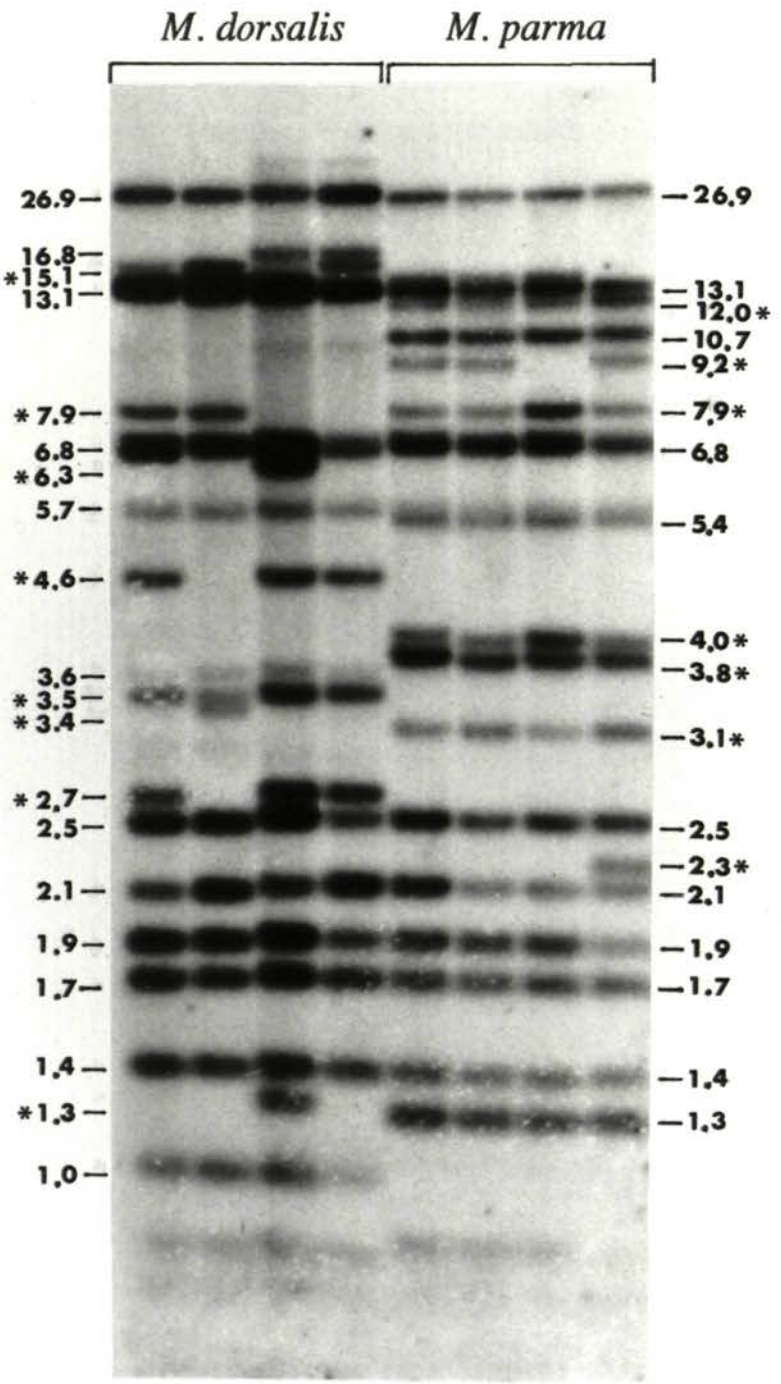
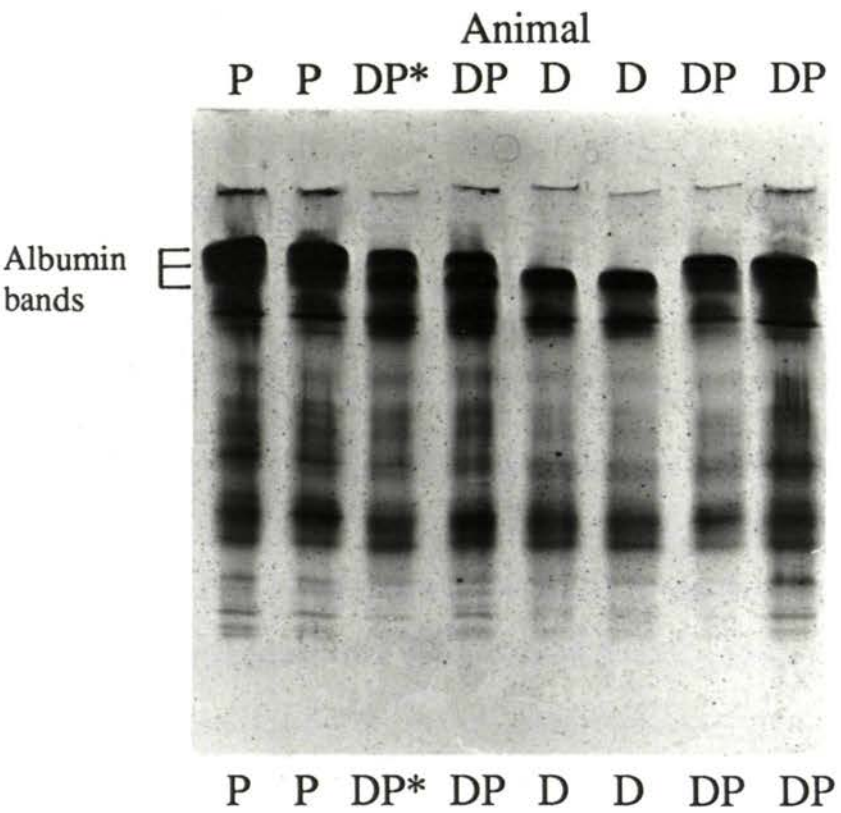

M. dorsalis
M. parma
26.9
16.8
*15.1
13.1
*7.9
6.8
*6.3
5.7
*4.6
3.6
*3.5
*3.4
*2.7
2.5
2.1
1.9
1.7
1.4
*1.3
1.0
26.9
13.1
12.0*
10.7
9.2*
7.9*
6.8
5.4
4.0*
3.8*
3.1*
2.5
2.3*
2.1
1.9
1.7
1.4
1.3

Figure 7.2. Albumin typings for parma (P), black-striped (D); putative F1 hybrid originally from Perth Zoo (DP*) and putative backcross animals (DP).


Animal
P P DP* DP D D DP DP
Albumin bands
P P DP* DP D D DP DP
Phenotype

CHAPTER 8

DISCUSSION

8.1 BREEDING AND MAPPING

One of the central aims of this Ph.D. was to develop an efficient system for generating a map of a marsupial genome through recombination. As stated, the number of species for which genetic maps are being developed has increased substantially over the last decade (O'Brien 1993). To date the majority of available maps are for eutherian species. Comparison of eutherian and marsupial maps may help elucidate the evolution of genetic linkage groups and to identify ancient therian chromosome structure and organisation. In addition, comparisons between marsupials and eutherians may provide further understanding of gene regulation and expression in mammals. Development of a marsupial map has been hindered in the past largely due to lack of sufficient detectable genetic variation within the study species. In order to overcome this difficulty an approach of crossing genetically distinct wallabies was employed. Two different systems were assessed for their usefulness. The first was an interspecies cross between the parma wallaby and its closest relative the black-striped wallaby. The second was an intraspecies cross between different island populations of the tammar wallaby. The wider the cross, that is, the greater the genetic distance (D) between the parental strains, the greater potential for detecting large gene frequency or fixed differences between them. With this in mind the interspecies cross between parmas and black-striped wallabies seemed to have a greater potential for usefulness in developing a linkage map. A further consideration of the potential usefulness of wide crosses is that the wider the cross, the more likely are difficulties in successful interbreeding. Parmas and black-stripes are known to hybridise in captivity (Close and Lowry 1990). However, attempts at producing F1 hybrids between parmas and black-stripes over several

years were unsuccessful. So whilst matings between the two are known to occur, the species do not hybridise readily enough to be useful for linkage mapping.

The second system of crossing two subspecies of tammar wallabies, one from KI in SA and the other from GI in WA was successful. This cross was thought to have potential for three reasons. Firstly, previous studies based on allozyme and protein data indicated that the two populations were genetically distinct (van Oorschot and Cooper, 1988a). Secondly, F1 hybrids had been successfully bred at CSIRO Division of Wildlife and Ecology, Canberra and both sexes were known to be fertile. Thirdly, reproduction in female tammars can be manipulated so that each female can, theoretically, give birth to 4-5 offspring per year (Hinds et al., 1990). Hence, it seemed likely that large numbers of progeny could be bred in a relatively short period of time. Crosses were set up to produce F1 hybrids and these animals were then backcrossed to the parental strains. Consideration of breeding information gathered over four years indicates that there is the beginnings of a barrier to reproduction between the two subspecies at least in the first year of running them together. A number of F1 hybrids were produced however, but not as readily as within subspecies progeny. There was no evidence of a barrier to reproduction between F1 hybrid animals and the parental strains. Hence, once sufficient F1 hybrids are produced the system is as efficient as breeding within subspecies tammars.

The level of genetic diversity between the two subspecies was assessed using allozyme, protein and RFLP markers. Whilst differences were detected between the KI and GI using allozymes and proteins, the efficiency of identifying genetic differences between the populations was significantly increased by the use of RFLPs. Seventy five percent of DNA probes used showed fixed differences between KI and GI tammars. Markers which showed large gene frequency of fixed differences between the populations were used to type backcross progeny. Of the fifteen markers used, seven belonged to linkage groups. The power of this system is evident. Over a three to four year period sufficient numbers of test-cross progeny have been produced and high level genetic diversity between the two populations can be

efficiently detected. As more markers are used the possibility of detecting linkage will increase.

Additionally, as F1s of both sexes are fertile, it is possible to generate linkage data based on male and female recombination frequencies. Comparison of male and female recombination frequencies in the tammar show that there is either no sex differences or that there is significantly higher recombination rates in the female than the male, similar to the situation observed in eutherians. This is in stark contrast to two other marsupial species *Sminthopsis crassicaudata* and *Monodelphis domestica* where there is severely reduced female recombination. More information from additional species is needed before the evolutionary significance of sex differences in recombination becomes clear.

Previous reports of very low levels of female recombination rates in marsupials suggested that linkage mapping of the X-chromosome in marsupials could be difficult if not impossible. Data on female recombination rates in the tammar indicates that mapping the X-chromosome, at least in this species, is quite possible. That female recombination rates in tammars are similar to or greater than male recombination rates indicates that it is very likely to be possible to generate a linkage map of the X-chromosome in this species.

Sex differentiation in marsupials is more complex than in eutherians, with evidence of the involvement of the X (Sharman et al., 1990). More specifically, it has been proposed by Cooper (1993) that sex determination in marsupials is influenced not only by the presence/absence of a Y chromosome as in eutherians, but also by the number of X chromosomes present and the parental origin of these chromosomes. The tammar system described here will enable the correlation between map position on the X and studies of expression of paternally and maternally derived alleles. Also of considerable importance is that it will aid the identification of the parental origin of X-chromosomes in intersexual animals. This system provides a unique and powerful opportunity to understand more fully the underlying mechanisms of X-chromosome

inactivation and sex differentiation and determination in marsupials and their evolution in mammals.

8.2 OTHER THEMES

During the course of this Ph.D. data was generated which enabled several issues regarding specific conservation genetics questions to be addressed in the tammar wallaby and the parma wallaby. The conclusions drawn from these data will now be discussed.

8.2.1 Conservation

Of the marsupials, the KI population of the tammar wallaby is, with *M. domestica*, the most investigated. Much is known of its reproductive biology, physiology, anatomy, immunology and genetics. As such, it has been proposed that the tammar wallaby has become the reference species for macropodids (Hinds et al., 1990), if not marsupials in general. KI tammars are the only extant, naturally occurring population in SA. There are also populations of five WA islands and a dwindling population in southwest WA. The tammar wallaby is listed as potentially vulnerable to extinction (Kennedy 1992).

Whilst there is a great deal of literature regarding the KI population, relatively little is known of the other populations. A small number of comparative studies indicate that there are significant differences in their biology in addition to the apparent beginnings of reproductive isolation between the WA and SA populations. These differences and calculation of the genetic distance between KI and GI supports suggestions that the two populations may be different species. This has implications for conservation strategies for the tammar. It is likely that each population will need to be managed as a separate entity. As a result of the mapping study, data was generated regarding the level of genetic variability within KI and GI. Garden Island tammars form a small isolated population. It was of concern that this may have led to a loss of heterozygosity. Comparison of the level of genetic variation between KI and GI animals indicates, surprisingly, that this is not the case. Additionally, there is no

significant difference between the level of genetic variability in KI and GI tammars in comparison to sheep and humans.

Similar data generated during investigations into the usefulness of parmas and black-striped wallaby crosses for mapping studies allowed for some important conservation questions relating to the parma wallaby to be addressed. The parma wallaby's range has been restricted and fragmented by agriculture and logging. They are now only found in small, widely separated populations. In fact, they were thought to be extinct on the Australian mainland until 1974. The survival of the parma wallaby could depend on captive breeding colonies and reintroduction programs. A large expatriate population of parmas is found on Kawau Island in New Zealand. Most captive colonies are derived from there. It was of concern that the New Zealand derived parmas might not be suitable for reintroduction programs for the following reasons: i) as founder numbers are unknown the New Zealand population could have a restricted genetic base, ii) as the origin of these animals is unknown, it was possible that they may be genetically distinct from the remaining natural populations, and iii) there was concern that black-striped wallabies might have hybridised with parma wallabies on Kawau Island affecting the genetic integrity of the parmas. Genetic typing of New Zealand derived parmas, parmas from the Australian mainland and black-striped wallabies has shown that New Zealand derived parmas do not have a restricted genetic base and are not genetically distinct from the remaining Australian parmas. There was also no indication of introgression between black-striped wallabies and parma wallabies in New Zealand.

8.2.2 The Major Histocompatibility Complex

A cDNA probe, Maru-DBB, which contained marsupial MHC class II sequence, was used initially as part of the mapping study, simply as a marker. However, close inspection of the results of Southern hybridisation with this probe showed an unexpected and intriguing result.

Eutherian MHC class II loci show high levels of polymorphism. In contrast, there is extremely low levels of variability at these loci in the tammar wallaby.

Comparison of data from the tammar with data from a number of eutherian species shows that the level of MHC variation in this marsupial species falls between that found in cheetahs and a population of lions, both of which have a restricted genetic base. There is no significant difference between the average heterozygosity found in tammars compared with sheep and humans. Hence, low MHC class II variation in tammars is not a reflection of an overall decrease in genetic polymorphism in this species. This is supported by data presented in Chapter 4 which shows that the average heterozygosity of class II loci is approximately 50% less than the overall level of heterozygosity in both KI and GI tammars.

Difficulties in detecting mixed lymphocyte culture responses in all marsupial species studied had led to the suggestion that marsupials have a class II system which exhibits limited variation. This has now been supported by data presented here.

There have been suggestions that genetic variability at MHC loci plays a role in successful reproduction. One of the most striking differences between eutherians and marsupials is their modes of reproduction. Although marsupials have placentae, they are generally shorter lived and less invasive than in eutherians. It is tempting to speculate that the differential MHC class II variation in marsupials and eutherians is connected to their respective modes of reproduction, although it is difficult, in the present state of knowledge, to propose a specific mechanism.

8.3 FUTURE DIRECTIONS

Interest in studying mammalian genome organisation and evolution has gained considerable momentum over the last decade. There are now genetic linkage maps available for many species (O'Brien 1993), the majority of which are eutherian. A map of a marsupial genome would increase our understanding of these important biological questions substantially.

The system described here, of crossing genetically distinct populations of tammar wallabies, is the most efficient available for generating a marsupial genetic map through recombination. Already comparisons can be made between the

organisation of the tammar genome and those of four eutherian species. As the tammar map grows, so will its potential for studying specific aspects of genome organisation and evolution and gene expression, such as

i) further detailed comparisons between marsupial and eutherian linkage groups

ii) understanding the distribution and significance of differential recombination rates in male and female tammar wallabies

iii) the underlying mechanisms of X-chromosome inactivation

iv) the origins and maintenance of sex differentiation and determination in marsupials and their evolution in mammals.

As the tammar wallaby becomes increasingly important as a model or reference species, it is clear that detailed investigations comparing the different populations are warranted. This is important both from a conservation perspective and also from a scientific perspective. For example, studies of the reproductive cycle of female tammars from Middle Island in Western Australia may increase our understanding, not only of tammar reproduction, but also of the mechanisms of marsupial reproduction as a whole. Further investigations into the effects of isolation on small populations of tammar wallabies will be of great importance in formulating conservation policy regarding endangered macropodid species.

There are two final points I would like to make. Firstly, the data presented in this thesis reinforces sentiments expressed by others, that one cannot assume aspects of eutherian biology will also be true for marsupials. Levels of genetic variability at MHC class II loci are a case in point. Secondly, marsupials are an enormous yet under exploited resource in efforts to understand mammalian evolution.

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