Fine-scale population genetics studies of koala populations in New South Wales and Victoria.

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Abstract

Mammal populations around the world are increasingly threatened with population fragmentation due to loss of habitat or barriers to gene flow. The fine-scale investigation of koala populations in Australia not only provides valuable information about this vulnerable species, but also serves as a model for population genetic studies in other species. The two studies in this thesis use Bayesian assignment methods and genic frequency analysis methods to identify demographically separate populations and barriers to gene flow between those populations.

In *Chapter 3*, three discrete populations were resolved near Sydney, New South Wales, with all displaying moderate to high levels of genetic differentiation among them (θ = 0.141–0.224). The allelic richness and heterozygosity of the Blue Mountains population (A=6.46, H_O=0.66) is comparable to the highest diversity found in any koala population previously investigated. However, considerably lower genetic diversity was found in the Campbelltown population (A=3.17, H_O=0.49), which also displayed evidence of a recent population bottleneck (effective population size estimated at 16–21). In addition, animals separated by a military reserve were identified as one population, suggesting that the reserve maintains gene flow within this population. By contrast, strong differentiation of two geographically close populations separated by a number of potential barriers to gene flow suggested these land-use features pose barriers to gene flow. These results indicate the need to carefully consider the future of the military reserve, along with the merits of assisting gene flow across the proposed barrier regions. Since these are demographically separate populations, specific management plans tailored to the needs of each population should be formulated.

In *Chapter 4,* koalas in the South Gippsland region of Victoria, Australia, were examined using microsatellite markers to infer population structure and gene flow and to locate a possible remnant gene pool. The results indicate that the South Gippsland koala population had higher genetic diversity (A=5.97, HO=0.564) than other published Victorian populations, and was genetically distinct from other koala populations examined. The study also indicates that South Gippsland koalas most likely survived the population reductions of the koala fur trade and now represent a remnant Victorian gene pool and should have a specific and tailored conservation management program.

The two research papers that form this thesis are two of the most comprehensive studies to date of koala populations using genetic methods at such a local level. The studies may serve as a reference to other research groups intending to investigate the population genetics of mammal populations spread over significant geographic distances.

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Any errors or inadequacies that remain in this work are entirely my responsibility.

Declaration

This work has not been previously submitted for a degree or diploma in any University. To the best of my knowledge and belief, the thesis presented is original and contains no material previously published or written by another person except where due reference is made in the thesis itself or noted below. This thesis contains two research papers that have been published in peer-reviewed journals. I am lead author on both papers, and the contribution of co-authors is noted below:

Chapter 2: **Defining spatial genetic structure and management units for vulnerable koala (Phascolarctos cinereus) populations in the Sydney region, Australia**

Dr Kyall Zenger provided initial direction and planning for the research and assistance in conducting laboratory work, along with performing an early supervisory role. Dr Zenger also offered advice on data analysis and interpretation and manuscript prearation.
Dr Robert Close contributed many samples and substantially assisted in the collection of other samples used in this research. Dr Close also assisted in data interpretation and manuscript preparation
Dr David Phalen supervised the project, assisted in laboratory work and data analysis and provided assistance in manuscript planning preparation and editing.
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Chapter 3: **Genetic analysis reveals a distinct and highly diverse koala (*Phascolarctos cinereus*) population in South Gippsland, Victoria, Australia**

Dr Kyall Zenger contributed to project planning and provided advice on laboratory work, data analysis and interpretation and contributed to manuscript editing.
Dr Robert Close assisted in data interpretation and manuscript preparation.
Dr David Phalen supervised the project, assisted in laboratory work and data analysis and provided assistance in manuscript planning preparation and editing.

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Introduction

The koala, *Phascolarctos cinereus*, is a charismatic Australian native species, recognised throughout the world. Although 'iconic' Australian marsupials, koalas have experienced a turbulent history, and many populations have been impacted by localised extinctions, population bottlenecks, disease, reintroductions and overpopulation, all of which can affect the health and viability of these animals (ANZECC 1998, Houlden et al. 1999c, Phillips 2000, Masters et al. 2004, Cristescu et al. 2009). Koalas play important roles in promoting conservation issues, as an attraction for Australian tourism industries, and as ambassadors for Australia. Koalas also play a role as a 'flagship' species for conservation (Gordon and Hrdina 2005); by capturing the public's attention, imagination and appeal, they can promote conservation issues, protection of forests and ultimately benefit a wide variety of native species. The koala is also economically important to Australia, and through its contribution to Australian tourism and advertising, its value to Australia's economy has been estimated at over $1.1 billion per year (Hundloe and Hamilton 1997).

European settlement in Australia and the koala fur trade

Koala numbers at the time of European settlement are difficult to estimate, although early reports from historians, settlers and naturalists can assist with estimation (e.g. Lee and Martin 1988, Phillips 1990, Jackson 2007). In general, historical records indicate koalas were rarely seen at the time of European settlement. Indigenous Australians hunted them for food and clothing and it is thought this reduced koala numbers to the extent that koalas were not documented until 10 years after the first European settlements (Jackson 2007). The apparent failure to observe koalas within the Sydney basin during a period of rapid exploration and colonisation suggests that koala numbers were very low. As the European population increased, the Aboriginal population's traditional way of life was disrupted, and this apparently coincided with an increasing koala population, presumably caused by decreased hunting pressure. Koala numbers eventually increased to the extent where a substantial fur trade became viable and global demand proved strong (Serventy and Serventy 1989, Melzer et al. 2000, Gordon and Hrdina 2005).

Koala hunting and fur export occurred over a period of up to 30 years in some parts of Australia (Serventy and Serventy 1989, Gordon and Hrdina 2005), from the late 1800's to early 1900's. The number of koalas killed for their furs during the koala fur trade indicates koalas must have been abundant and widespread across Eastern Australia during this time; some records state that between 1919 and 1924 alone, eight million koalas were killed and their pelts exported (Martin and Handasyde 1999). The koala fur trade resulted in rapid population declines and localised extinctions across Australia and was likely responsible for koalas becoming extinct in South Australia and much of Victoria (Phillips 1990). Numbers in Victoria were estimated to have fallen to between 500 and 1000 as a result of the fur trade (Warneke 1978). Towards the end of the fur trade, a small number of koalas was moved to French Island off the coast of Victoria in an apparent effort to save koalas

from extinction (Martin and Handasyde 1999). Anecdotal evidence suggests the French island population may have been established with as few as two or three individuals (Martin and Handasyde 1999). Genetic analysis has revealed severely reduced genetic diversity of French Island animals with only two mitochondrial DNA haplotypes discovered (Taylor et al. 1997a).

Following the end of the fur trade in the late 1920's, koalas were gradually restocked onto the mainland of Victoria through a State government-run translocation program, using animals from the bottlenecked French island population (Houlden et al. 1996). Although successful in increasing numbers of koalas on the mainland, the translocation program is responsible for the relatively low genetic diversity seen in many Victorian koala populations today (Timms et al. 1993, Houlden et al. 1996, Taylor et al. 1997a). Victorian populations derived from translocated animals have an average reported allelic diversity of around 3 alleles/locus (Houlden et al. 1996) compared to populations in NSW which have up to 6.83 alleles/locus (Lee et al. 2010b and Chapter 3). Population bottlenecks such as that created by establishing French Island with few founder animals, reduce genetic diversity, which limits the ability of the population to adapt to change and increases the chance of congenital defects (Bruford et al. 1996, Frankham et al. 2002).

In addition to the lasting effects on koala population size and genetic diversity, the fur trade also had a profound effect on Australian society's attitude towards koalas. Public outrage at the mass slaughter eventually led to southern states gradually introducing legislation making the koala fur trade illegal; although open seasons and export continued in Queensland (Jackson 2007). When the United States of America prohibited import of koala skins in 1930, the main export market was closed and the koala fur trade was effectively over. Three years later, after over three decades of inaction and unwillingness to use legitimate constitutional powers over customs and exports, the Australian Government passed legislation banning the export of koala products in Australia, legally and formally ending the koala fur trade.

Despite the effects of the fur trade, koalas in some areas have shown the ability to successfully recover (Masters et al. 2004, Duka and Masters 2005). Koalas in areas of Victoria and on Kangaroo Island, South Australia are now considered to be overabundant and active management is necessary to prevent starvation due to defoliation of koala food trees (Masters et al. 2004, Duka and Masters 2005). Culling is politically impractical, and consequently sterilisation followed by translocation to the mainland is the current practiced management option. Koala populations in Queensland and New South Wales (NSW), however, have been slower to recover and in recent years some populations have continued to decline while others have become extinct altogether (Reed et al. 1990, Phillips 2000, Preece 2007, Lee et al. 2010a). With overpopulation in some areas and declining populations in others, koala management in Australia is a complex and controversial topic, requiring specific plans on a local scale, rather than a uniform national approach. Therefore, specific populations need to be studied to understand their threats, and create tailored management plans.

Koalas have a widespread distribution in Australia, and are found along much of the east coast (Figure 1). However, their distribution is apparently highly fragmented, and ranges from the tropical north to the temperate south of eastern Australia. Koalas are distributed across the Great Dividing Range but not found in areas receiving regular winter snowfall.

Legal Status

The contemporary koala debate is centred on determining the appropriate legal conservation status. The legal conservation status varies between States and Territories; in NSW the koala is listed as vulnerable under the *Threatened Species Conservation Act 1995*; in Queensland the koala is listed as 'least concern' under the *Nature Conservation Act* 1992 (except in south east Queensland where it is listed as 'vulnerable to extinction'); while in Victoria the koala is not listed. On a national level, and in addition to any state laws, the koala is afforded Australian Government protection through the Australian Government's *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). Under the EPBC Act koalas in NSW, the Australian Capital Territory (ACT) and Queensland are listed as vulnerable. A vulnerable listing means any action (e.g., development/land clearing) that is expected to have a significant impact on koalas in NSW, ACT or Queensland must be referred to the Australian Government for assessment. The March 2012 listing of the koala under the EPBC Act affords substantial protection to koala populations, in addition to protection already received under State government laws. The koala's listing under the EPBC Act is particularly noteworthy given it is the first time a species has been listed and protected in certain geographical areas only (in this case Queensland, ACT and NSW) under the EPBC Act. In other species listed under the EPBC Act, the species is given protection regardless of where in Australia it is present. This anomaly recognises the difficulty in treating all koala populations equally and in doing so acknowledges a central assertion of this thesis – that specific koala populations have their own, and in some cases unique, characteristics and therefore require specific study.

Biology of the koala

The koala (Class: Mammalia; Subclass: Marsupialia; Order: diprotodontia; Suborder: vombatiformes) is the only extant member of the family Phascolarctidae. Koalas feed almost exclusively on leaves of the *Eucalyptus* species. Eucalyptus spp. are abundant across most of the east coast of Australia and dictate the geographic distribution of koala populations in Australia. Individual koalas often have particular tree preferences, although these are highly variable between regions and koalas (Hindell and Lee 1990, Moore and Foley 2005).

Classifying koalas into subspecies is a somewhat arbitrary process. Species populations generally exist as a continuum of gene flow across a given geographical area. Koalas have an extensive and varied natural range and, unsurprisingly, show significant regional variation. In general, koalas in Queensland are smaller in size and weight and lighter in colour than koalas from further south, and this distinction increases further south (Melzer 1994). Currently, koalas are classified into subspecies based on Australian State Government boundaries.

Koalas occurring in Queensland have been classified in the subspecies *P. c. adustus*, in New South Wales they are *P. c. cinereus*, while Victorian and South Australian koalas are *P. c. victor*.

The koala breeding season lasts from approximately October to March (White and Kunst 1990, Dique et al. 2003). During this breeding season adult males move more frequently, over greater distances and have larger home ranges (Mitchell 1990). Koalas are generally solitary (Mitchell 1990b), but social dominance appears to exist with dominant males apparently able to maintain a home range with access to females (Mitchell 1990a, Mitchell 1990b). However, dominance does not necessarily lead to monopolisation of mating opportunities; one study using genetic techniques concluded that 'resident' status confers no advantage for parentage and fathering is performed by both 'resident' and 'transient' male koalas (Ellis et al. 2002). Sexually mature females are able to produce one young per year, and indeed most Chlamydia-free females reproduce each year (Martin and Handasyde 1990). However, Chlamydia infection can markedly reduce fertility (see discussion at 4.3 below).

Female young typically establish a home range near their mother's home range, while males generally disperse greater distances and establish a home range by two to three years of age (Mitchell and Martin 1990b). Dique et al. (2003) conducted a comprehensive radio-telemetry study of dispersal patterns of 195 koalas at three sites in south-east Queensland. In this study, a significantly higher proportion of young males (72%) dispersed from the study site than females (35%). All dispersals in this study began between June and December, with more than 50% of males moving in July-August, and more than 50% of females moving in September-November. Mean distances dispersed were similar for both sexes (3.5km for males versus 3.4km for females), and there was quite a substantial range in dispersal distance, males ranging from 1.1 - 9.7km and females from 0.3 – 10.6km. Other studies of dispersal generally found a higher proportion of males disperse compared to females (Mitchell et al. 1988, Mitchell and Martin 1990a). However, Ramsay (1999) found dispersal rates that were roughly similar between the sexes (82% vs 92% for males and females respectively) in a population at Nowendoc, NSW. These results show that for most aspects and measures of dispersal and home range, koalas are remarkably variable. The consistent findings are that most dispersal occurs before koalas are three years old and dispersal is generally a spring and early summer activity.

Population genetics of the koala

Genetic markers for population studies

Microsatellites are ubiquitous throughout all nuclear eukaryotic genomes and are one of the most popular markers for conservation genetics studies due to their relative ease of use and versatility (Storfer et al. 2010). Microsatellites are tandem arrays of a short simple sequence of one to five base pairs in length. The high variability in these lengths makes microsatellites ideally suited to genetic studies. Microsatellites can be used to estimate effective population size, inbreeding coefficients (the chance of two alleles being identical by descent), migration between populations, genetic diversity and paternity (Luikart and England 1999).

Microsatellites have many favourable characteristics that make them well suited to population genetic studies: they show a Mendelian inheritance, are highly variable and are easily PCR assayable (Bennett 2000). Traditional methods for finding microsatellites and developing appropriate primers involved cloning and construction of DNA libraries. These methods are time consuming and expensive. New sequencing technologies can enable rapid identification of a large number of microsatellite markers (Abdelkrim et al. 2009), and are likely to enable rapid, cheap and efficient identification of microsatellites in future. The majority of conservation genetics studies on koalas have been conducted with microsatellite markers (see discussion below), including the two studies in this thesis.

Mitochondrial DNA haplotypes were used as one of the earlier markers for investigating populations, although they have now fallen out of favour compared to other markers with broader applications such as microsatellites. Mitochondrial DNA is limited due to lack of variability, especially in koalas (Tsangaras et al. 2012), and inheritance only through the maternal line (Murtskhvaladze et al. 2012). These characteristics mean that mitochondrial DNA has limited application to examining contemporary genetic structure or population genetic diversity, although may be useful when investigating evolutionary timescales or phylogeny.

Single Nucleotide Polymorphisms (SNPs) are single base changes along DNA sequence and have become particularly useful markers for population genetic studies. SNPs are particularly useful for scanning large regions of a genome due to their abundance and co-dominant nature (Williams et al. 2010), low scoring-error rates, and relative ease of calibration between laboratories compared to length based markers (for review see (Morin et al. 2004). Population genetic studies have used SNPs to identify differences between species (Primmer et al. 2002), populations (Weir et al. 2005), and individuals (Gill 2001).While microsatellites display greater allelic diversity per locus, when enough SNPs are used together they can detect population structure and in some cases individual SNPs will segregate strongly among populations (Freamo et al. 2011). The statistical power of approximately 100 SNPs is roughly equivalent to 10-20 microsatellites (Kalinowski 2002).

Analysis methods for studying population genetics

Most analysis of genetic data is conducted with specially developed population genetic computer programs, generally employing either the traditional 'frequentist' probability methods or more recent Bayesian modelling. The two studies in this thesis use a range of programs that employ both of these methods. Essentially, frequentist analysis involves more traditional probability, p-values and hypothesis testing, and examines the expected long term frequency of an observation (Allendorf 2007); whereas Bayesian approaches are model-based and combine likelihood calculations with prior information to arrive at a probability of a particular parameter (Beaumont and Rannala 2004). Bayesian analysis used in studies in this thesis (e.g. through programs such as Structure and Geneland) have several significant advantages over the 'frequentist' methods. However, both play an important role and can be used together to gain a more comprehensive understanding of a population. A major advantage of Bayesian modelling over frequentist approaches for conservation genetics is that Bayesian modelling is more suited to decision making when data are limited or

incomplete (Allendorf 2007), as is generally the case in field based wildlife studies. Bayesian modelling is also a more effective way to deal with the interdependent parameters that arise when studying genetic data of populations (Beaumont and Rannala 2004).

Amongst the Bayesian programs used in this thesis, Geneland distinguishes itself by incorporating spatial information into modelling. The location in which each individual was sampled is modelled alongside genotypic data and the Geneland model assigns greater probability to genetic clusters that are continuous within the spatial landscape (Guillot et al. 2005). Also, unlike Structure, Geneland does not require *a priori* estimation of the number of populations. Geneland instead allows the number of genetic clusters/populations to vary within the model and is able to estimate the most likely number of populations based on the data. Structure requires the number of populations to be defined before analysis and the process of estimating the most likely number of clusters is somewhat subjective (Evanno et al. 2005, Pritchard et al. 2007). Geneland's ability to analyse spatial data means it is ideally suited to the burgeoning field of landscape genetics, i.e. the study of how geographical and environmental features influence genetic variation at both the population and individual levels (Storfer et al. 2007).

Population genetics of koalas in Australia

Despite the complex issues surrounding koala management and conservation, there have been few genetic studies into koala population structure in Australia (Houlden et al. 1996, Taylor et al. 1997a, Houlden et al. 1999a, Fowler et al. 2000, Sherwin et al. 2000, Lee et al. 2010a). These studies have utilised morphological markers, microsatellites, allozymes and mitochondrial DNA, and combinations of such genetic markers.

The majority of population genetic studies have been performed on a broad scale, comparing a small number of koalas from a large number of geographically distant populations (e.g. Houlden et al. 1996). These studies have provided insights into the genetic variation and structure of koala populations on a national scale. Taylor et al. (1991) conducted one of the initial genetic investigations of koala populations when they examined the genetic variability of south-eastern Australian koalas using restriction enzyme digestion. Levels of variability were low and this was attributed to the perturbed history of the populations surveyed. Studies of a limited number of wild Queensland koalas using M13 probes revealed similar levels of variation (Timms et al. 1993).

Many of the published studies of koala genetics have used mitochondrial DNA (mtDNA). These studies have found low levels of mtDNA variability (Taylor et al. 1997b, Houlden et al. 1999b, Fowler et al. 2000). The low mtDNA variability could be a result of long-term population expansion and contraction, but this observed characteristic could also relate to limited female dispersal. Fowler et al. (2000) used mitochondrial DNA in a study on Queensland koala populations and concluded that female-mediated gene flow is likely to be limited, and noted the need for further work to investigate variation in nuclear DNA. Taylor et al. (1997b) used mitochondrial markers to study koala populations in Victoria, and found very low polymorphism, and

concluded this was consistent with the expectation that extensive human-mediated translocations had homogenised the Victorian koala population.

Despite the low variability, found using mtDNA, mitochondrial markers have found differentiation between populations: Houlden et al. (1999a) used mitochondrial markers to study over 200 animals from 16 populations throughout the koalas' range, and discovered significant differentiation in mitochondrial haplotype frequencies between localities suggesting that little gene flow exists among the studied populations. Houlden et al. (1996) also studied the Campbelltown population, featured in Chapter 2 of this thesis, and identified Campbelltown as a distinct management unit, finding six mitochondrial DNA haplotypes that were not present in other populations studied. Lee et al. (2010a) used both microsatellite and mitochondrial markers to study koala populations in southeast Queensland. Their mitochondrial data suggested that historically there was gene flow between koalas along the coast. However, the microsatellite data showed nearby populations to be genetically distinct, further suggesting that more recent gene flow has been heavily restricted, potentially due to increased urbanisation and reduced koala population size in the area (Lee et al. 2010a).

Limitation of previous population genetic studies of koalas

There are two main limitations to previous population genetics studies of koalas: the confounding influence of undocumented human-mediated movement; and the lack of geographically-close, fine-scale analysis. Human-mediated movement of koalas can confound historical patterns of genetic variation. For example, studies of Victorian koalas consistently find little genetic structure between populations over expansive geographical areas (eg. Houlden et al. 1996, Taylor et al. 1997b, Phillips 2000). Results of population genetic studies of koalas in Victoria likely reflect recent patterns of human-mediated dispersal, rather than 'natural' patterns of genetic variation. The second limitation is a lack of fine-scale genetic studies. Aside from the two studies in this thesis, there is only one other study (Lee et al. 2010a) that has comprehensively sampled a number of geographically nearby populations with a method that is able to detect fine-scale differentiation, such as microsatellite markers. Previous studies looked at a much broader area and more isolated and distant populations and have typically used mitochondrial DNA. As a result, previous studies often could not gain detailed information regarding koala migration and gene flow, or the presence of barriers to gene flow. Previous studies have therefore had limited ability to contribute to population-specific conservation management.

Chlamydia

Koalas are susceptible to a number of infectious and non-infectious conditions (Wood 1978, Canfield 1990, Griffith et al. 2013), the most significant being chlamydial disease. Two major chlamydial associated diseases have been described in koalas: that effecting the eyes and the respiratory track ; and that effecting the urinary tract and the female reproductive tract (Brown and Grice 1984).

Chlamydia are obligate intracellular bacteria and chlamydiosis is considered to be an opportunistic infection of koalas (Ladds 2009). Two species, *Chlamydophila pecorum* and *C. pneumoniae* cause disease in koalas. *C. pneumonia* is typically associated with conjunctivitis and *C. pecorum* can cause urogenital diseases (Higgins et al 2005). While prevalence of chlamydial infection is quite variable across populations, ranging from 0 – 90% (Ladds 2009), the prevalence of clinical disease is often much lower than infection (Ladds 2009). This suggests that additional factors, potentially stress, crowding, or immunosuppression from other diseases, play a role in causing disease in koalas.

In terms of a population-scale threat, Chlamydia infection can reduce fertility in female koalas, reducing the fertility rate for a population, although not to the extent that population growth is prevented (Martin and Handasyde 1999). The stark effect Chlamydia can have on fertility is further demonstrated by the Martin and Handasyde (1990) study that reported population doubling times that varied from approximately 12 years for the Chlamydia-positive Raymond Island population to approximately 3 years for the Chlamydia-negative populations in Sandy Point, Victoria. In another study comparing Chlamydia-infected and Chlamydia-free populations in Victoria, koalas in a Chlamydia-free population (Red Bill Creek) continued to breed from season to season, whereas females from a Chlamydia-infected population (Brisbane Ranges) stopped breeding for at least one season before resuming breeding in a later season (McLean and Handasyde 2006). Koalas are the only native Australian mammals that commonly suffer chlamydial disease (Ladds 2009) and signs of chlamydial infection have been observed in wild koalas since the 19th century (Troughton 1941).

Chlamydia is not present in all koala populations. The Campbelltown population (Chapter 2) appears to be free of Chlamydia (R. Close). However, neighbouring populations to the south and northwest have shown evidence of Chlamydia infection. The South Gippsland population (Chapter 3), along with most Victorian populations, is thought to be free of Chlamydia (Phillips 2000).

Overpopulation and inbreeding

Koala populations in some densely populated areas of Victoria and South Australia are at risk of over browsing (Masters et al. 2004) and may be more susceptible to disease due to low genetic diversity (Cristescu et al. 2009). Overpopulation and inbreeding are of particular concern for koala populations on islands, as they have a small and finite amount of habitat and nowhere to disperse as crowding increases. Over browsing can lead to koalas consuming substandard feed resulting in malnutrition or starvation (Duka and Masters 2005). Inbreeding or low genetic diversity is another threat faced particularly by island populations, but also some Victorian populations that are derived from translocated island stock. The general negative effects of inbreeding include inability to respond to changes in environment and increase in genetic diseases (Bruford et al. 1996, Frankham et al. 2002). A number of conditions specific to koalas have been described in populations of low genetic diversity. Seymour et al (2001) found the incidence of testicular aplasia correlated positively with effective inbreeding coefficients, and may be a symptom of inbreeding depression in populations on

French Island and Kangaroo Island. However, Cristescu et al. (2009) studied French Island and Kangaroo Island populations and could not find a relationship between internal relatedness, an individual inbreeding coefficient, and koalas with testicular abnormalities, suggesting the condition may not be related to recent inbreeding. They speculate that the condition could instead result from a chance selection of founder individuals carrying alleles for testicular abnormalities, followed by a subsequent increase in these alleles' frequencies through genetic drift. In any case, low genetic diversity has, so far, apparently not reduced koala populations' ability to grow, and indeed some of the populations with lower genetic diversity have apparently high growth rates, Kangaroo Island and French Island being the most obvious examples. Although there is scant evidence to indicate koalas are currently suffering ill-effects of low diversity, it remains prudent to guard against future threats, such as climate change or disease, by employing strategies that aim for the maintenance of greatest genetic diversity.

Objectives and Scope of this project

Some aspects of ecology that cannot be easily observed can be inferred through genetic studies. In writing this thesis it was my overall aim to show how the study of genetics can be applied to individual populations and regions in order to inform management plans. The two papers in this thesis present fine-scale studies of genetic structure using microsatellite markers that can be used to inform conservation management. The papers describe genetic structure and gene flow between nearby populations, and show how this knowledge can inform and guide conservation management. Both papers also show how fine-scale genetic analysis can be applied to understand a population's demographic history. When this project was initiated there was very little published knowledge of the population structure or gene flow between populations surrounding Sydney. Knowledge of the population history and genetic structure of koalas in South Gippsland, Victoria, was also somewhat limited; what was inferred about South Gippsland koalas was based on anecdotal evidence and limited historical records supported by preliminary genetic studies. Previous studies of South Gippsland koalas were unable to draw strong conclusions, due to limited sampling schemes, the types of genetic markers used and the population-genetics analysis tools available.

Studying koala populations is challenging. The koala is a cryptic species and studies of low density populations are hampered by the enormous effort necessary for investigators to find even a few animals. As with other cryptic species, community participation can prove invaluable in ecologic studies, such as in sea turtles (Martin and James 2005), tamarins (Savage et al. 2010) and frogs (Somaweera et al. 2010). Community assistance including alerting researchers to chance sightings, completing surveys to provide historical records and active engagement in koala searches can provide information that could not be obtained by researchers in a similar period of time (Lunney et al. 1997, Crowther et al. 2009, Lunney et al. 2010). The koala is particularly suited to community assisted studies as it is a charismatic and well known species, is easily recognized and generates excitement and therefore prompt reporting when found. The two studies in this thesis would not have been possible without the assistance of the local communities.

Study sites

The Campbelltown koala population located due south of Sydney is an example of a low density semi-urban population descended from koalas that have survived the fur trade. Campbelltown koalas were harvested until the mid-1920's and were presumed to be extinct due to the lack of published observations of them, until 1986 when koalas were sighted in an area earmarked for development (Ward and Close 1998, Ward 2002, Ward and Close 2004). After community protest (and the first union 'green ban' to protect animal habitat) the development was abandoned and the local council rezoned the land as regional open space (Sheppard 1990). Following the discovery of this breeding population, a community assisted research program was instituted in 1990 that continues to the present (Ward and Close 1998). The history of the Campbelltown population is described in greater detail elsewhere (e.g. Sheppard 1990, Ward and Close 1998, Ward 2002, Ward and Close 2004). A preliminary survey indicated that a more comprehensive study was possible, leading to the initiation of a PhD study by Dr. Steven Ward in 1995. Dr. Steven Ward's 2002 PhD thesis provided a detailed introduction to the Campbelltown population (Ward 2002) and paved the way for studies, such as those contained in this thesis, to advance the work in greater detail and scope. This thesis therefore complements and expands a substantial body of work that has preceded it. Chapter 2, while focusing on the Campbelltown population, investigates koala populations in four nearby geographic regions: Campbelltown, Heathcote, Southern Highlands and Blue Mountains. These four regions represent all known koala-inhabited areas of the Sydney basin (Ward 2002). The habitat is eucalyptus forests, covering terrain varying from steep gorges dispersed throughout a plateau (Campbelltown and Heathcote sites), a rugged, steep, mountainous area (Blue Mountains populations) and a highlands region (Southern Highlands). The areas vary from peri-urban (Campbelltown and Heathcote) to agricultural (Southern Highlands) to well forested (Blue Mountains). The land to the north of Campbelltown and the Southern Highlands and to the east of the Blue Mountains is the heavily urbanised Sydney region. Koalas are distributed throughout the study sites. No reliable estimate exists for population size of the Southern Highlands or Blue Mountains populations. The Campbelltown population size has been estimated at 90-200 (Ward 2002) in 2002 and the population is expected to have increased since then (Close, unpublished data 2013). In 2010 the Australian Koala Foundation (AKF), in cooperation with the authors associated with Lee et al. (2010b) and Lee et al. (2012), published on the AKF website an estimate for the Campbelltown koala population of 260 koalas (AKF website, 2010).

The Campbelltown population is a potentially important population as it is Chlamydia free, the nearest population to Sydney, is apparently increasing in size and is relatively isolated (in terms of migration) from nearby populations. This isolation could be assisting the population to remain free of Chlamydia. Despite their importance the Campbelltown koalas face an uncertain future; as Sydney's population grows, pressure on the Campbelltown koala habitat will increase, available habitat is likely to decrease and threats from dogs and cars are likely to increase.

As described earlier in this introduction, Victoria was hit hard by the fur trade and near the end of the fur trade period a small number of koalas were moved to French Island. Anecdotal evidence also suggests a small

number of koalas remained in the steep and difficult terrain of the Strzelecki ranges in eastern Victoria and survived the fur trade. There have long been unsupported claims that the Strzelecki ranges could therefore hold a remnant gene pool, perhaps representative of Victorian koalas before the fur trade. The Victorian Government's Koala Management Strategy (DSE 2004) recognises a possible remnant genetic resource in South Gippsland koalas and states that a high priority is to conduct a "detailed survey of genetic diversity" across the South Gippsland region. Chapter three therefore seeks to determine if a remnant population did indeed survive the fur trade and remains to this day.

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Defining spatial genetic structure and management units for vulnerable koala (*Phascolarctos cinereus*) populations in the Sydney region, Australia.

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Abstract

Context: Mammal populations around the world are increasingly threatened with population fragmentation due to loss of habitat or barriers to gene flow. The investigation of Koala populations in the Sydney region not only provides valuable information about this vulnerable species, but also serves as a model for other species that have suffered major rapid declines in population size, and are now recovering in fragmented habitat. The peri-urban study region allows investigation of the impact of landscape features such as major roads and housing developments on koala gene flow.

Aims: Animals originating from four geographic sampling areas around Sydney, New South Wales, Australia were examined to determine population structure and gene flow and to identify barriers to gene flow and management units.

Methods: This study examined 12 microsatellite loci and used Bayesian assignment methods and genic frequency analysis methods to identify demographically separate populations and barriers to gene flow between those populations.

Key Results: Three discrete populations were resolved, with all displaying moderate to high levels of genetic differentiation among them (θ = 0.141–0.224). The allelic richness and heterozygosity of the Blue Mountains population (A=6.46, H_O=0.66) is comparable to the highest diversity found in any koala population previously investigated. However, considerably lower genetic diversity was found in the Campbelltown population (A=3.17, H_O=0.49), which also displayed evidence of a recent population bottleneck (effective population size estimated at 16–21).

Conclusions: Animals separated by a military reserve were identified as one population, suggesting that the reserve maintains gene flow within this population. By contrast, strong differentiation of two geographically close populations separated by a number of potential barriers to gene flow suggested these land-use features pose barriers to gene flow.

Implications: Implications of these findings for management of koala populations in the Greater Sydney region are discussed. In particular the need to carefully consider the future of a military reserve is highlighted, along with possible solutions to allow gene flow across the proposed barrier regions. Since these are demographically separate populations, specific management plans tailored to the needs of each population will need to be formulated.

Introduction

Mammal populations around the world are increasingly threatened with population fragmentation. Populations can become fragmented due to habitat loss or the presence of anthropogenic barriers to gene flow, such as major roads (Balkenhol and Waits 2009). If barriers are present in an important migration or dispersal corridor they could significantly impede gene flow across that region effectively isolating adjacent populations (Perez-Espona *et al.* 2008). Fragmentation and consequent isolation can reduce the genetic diversity and therefore the species ability to respond to environmental change (Frankham *et al.* 2002). In the context of species conservation, *management units* are generally recognised as demographically independent populations (Palsboll *et al.* 2007). The identification of management units is a crucial step in the management and conservation of natural populations (Palsboll *et al.* 2007).

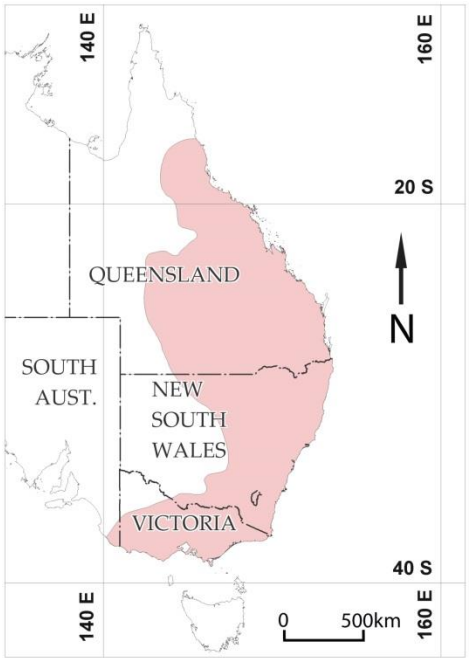

Figure 1 East coast map of Australia with shading indicating the broad geographical range of koalas. Koala populations in this range are highly fragmented. Reproduced with permission from the Australian Koala Foundation

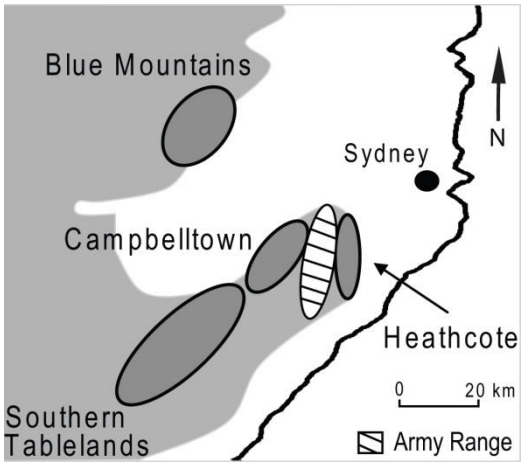

Figure 2 The greater Sydney region showing sampling locations (circled) and potential koala habitat (shaded).

The koala (*Phascolarctos cinereus*) is a valuable iconic Australian marsupial. It is internationally recognisable and plays a significant role in promoting Australian fauna awareness. The koala is also fundamentally important in its biological uniqueness. Koalas are unique in that they are the only extant member of the Family Phascolarctidae and one of the few mammals that feed almost exclusively on eucalypts. Despite this, koala populations have significantly declined over the years through hunting for the koala fur trade and habitat destruction from human activities (Hume 1990; Reed and Lunney 1990). Along with many other mammal species, increasing urban sprawl has caused koalas to be increasingly threatened by factors such as motor vehicles, loss of habitat and dog attacks (Dique *et al.* 2003; Lunney *et al.* 2004). The current geographical distribution of the koala ranges across much of the eastern coast of Australia (Figure 1).

Koalas were believed to have been lost from large areas surrounding Sydney (Reed *et al.* 1991), but recent sightings indicate that some populations are gradually expanding back into the remnants of their original range (Ward and Close 2004). . The Sydney koalas may not have experienced the same degree of genetic bottleneck that occurred in Victorian koalas and were not subjected to translocation programs as seen in Victorian koalas (Houlden *et al.* 1996b). Consequently, the Sydney koalas may retain genetic characteristics not found in other koala populations. Additionally, there have been no studies into koala population genetic structure within the Sydney basin. Management of these populations may therefore not be optimal because knowledge of population structure is necessary when developing management plans for scattered remnant populations (e.g. Manel *et al.* 2003; Storfer *et al.* 2007). Koalas also occupy some of the remaining undeveloped land in Sydney's south-west, and therefore careful development planning will be needed in order to maintain healthy koala populations while allowing for Sydney's growing population, which is projected to increase by approximately 63 % over the next 50 years (ABS 2006). This paper aims to investigate the current population structure of koala populations surrounding the Sydney region in order to provide information to better manage this species. In addition, this study may also offer insights into factors that may affect other animal species in similar urban environments that have also suffered major rapid declines in population size in the past but are now recovering.

The koala's ability to recover from threatening processes and population declines has varied over its geographical range. Koalas in many areas of Victoria and on Kangaroo Island, South Australia are now considered to be overabundant (Masters *et al.* 2004). However, in New South Wales (NSW) and Queensland, some koala populations have continued to decline or have become extinct altogether (Reed *et al.* 1991; Phillips 2000). With overpopulation in some areas and declining populations in other areas, koala management in Australia is a complex and controversial topic (e.g. Phillips 2000) that requires specific management plans at a local scale, rather than a single uniform national approach.

Despite the complex issues surrounding koala management and conservation, there have been few genetic studies into koala population structure in Australia (Houlden *et al.* 1996b; Taylor *et al.* 1997; Houlden *et al.* 1999; Fowler *et al.* 2000; Sherwin *et al.* 2000). When comparing microsatellite data from ten populations around Australia, Houlden et al. (1996b) found significantly lower levels of intra-specific population differentiation among Victorian koala populations, compared to northern NSW and Queensland populations. They concluded that this reflects the extensive human perturbation in most Victorian populations. Taylor et al. (1997) using mitochondrial DNA data supported these findings. Fowler et al. (2000) used mitochondrial DNA in a study on Queensland koala populations and concluded that female-mediated gene flow is likely to be limited, and noted the need for further work to investigate variation in nuclear DNA.

Landscape features, both natural (e.g. rivers, mountains) and artificial (e.g. roads, housing) can be barriers to gene flow for certain species depending on their methods of migration. Several putative barriers exist between the sampling regions in this study, and are investigated to determine if they have any affect on gene flow between the two sampling regions. One hypothesised barrier region is transected by a major arterial road that may impede koala dispersal. A two kilometre stretch of this road has previously been identified as a koala fatality blackspot (Ward 2002). In addition, this area is surrounded by an urban area, a colliery, steep river tributaries and contains habitat not considered prime koala habitat. These factors might all combine to create effective barriers to dispersal.

Two sampling regions in this paper are separated by the 18000 hectare Holsworthy Army Range, which lies approximately 30 kilometres from the Sydney central business district, and has been in active use since approximately 1914. The future of the Holsworthy Army Range is currently uncertain and therefore it is necessary to examine whether the range provides connectivity between the two sampling regions.
There is a mix of habitat within the area, some of which would be expected to support koalas. However, koala presence within the area is difficult to confirm due to restricted access. One ear-tagged young male koala originally observed and tagged within the Campbelltown sampling region has been sighted near the northeastern border of the Army Range (Ward 2002), indicating that males at least are able to move through some of the range. While there are no specific reasons to assume koalas do not inhabit the range, the uncertainties regarding koala status, combined with the potential importance of the land and the uncertain future, deem the subject to be worthy of further investigation.

The objectives of this study were to help guide management decisions aimed at conserving koala populations inhabiting the rapidly changing environment in the Sydney region. This paper seeks to use genetic information to define management units for koala conservation. Potential natural and anthropogenic barriers are also investigated with an aim to determine if they impede gene flow. This paper will also aim to determine the importance of the Holsworthy Army Range to local koala populations, and consequently to inform future land uses decisions within the Army Range. To achieve these objectives this paper uses 12 microsatellite loci to investigate population structure, genetic diversity and barriers to gene flow in remnant Sydney koala populations.

Materials and Methods

Study area

Samples were obtained from four geographic regions: Campbelltown (n=101), Heathcote (n=9), Southern Tablelands (n=16) and Blue Mountains (n=18) for a total of 144 samples (Figure 2). These four regions were selected because they represent all known koala-inhabited areas of the Sydney basin (Ward 2002). The habitat is eucalyptus forests, covering terrain varying from steep gorges dispersed throughout a plateau (Campbelltown and Heathcote sites), a rugged, steep, mountainous area (Blue Mountains populations) and a tablelands region (Southern Tablelands). The areas vary from peri-urban (Campbelltown and Heathcote) to agricultural (Southern Tablelands) to well forested (Blue Mountains). Although the Campbelltown and Southern Tablelands sampling areas are in close proximity, they are considered separate geographical regions for the purposes of this study because of their differing land uses and because the intervening landscape has several possible barriers to gene flow. The land to the north of Campbelltown and the Southern Tablelands and to the east of the Blue Mountains is the heavily urbanised Sydney region.

Sample collection and genotyping

Samples were collected during the period of 1998 to 2008. The koalas sampled were part of an ongoing study monitoring koala populations in the Greater Sydney region conducted by The University of Western Sydney and The University of Sydney. Samples were obtained opportunistically from deceased animals along roadways or from local field survey programs. Consequently, samples were more abundant from urbanised areas than from non-populated regions. A small number of samples was also collected as a result of deliberate expeditions aimed at sampling koalas to fill in spatial gaps. At the time of sampling, Universal Transverse Mercator (UTM) coordinates were recorded using a handheld global positioning device. The samples were collected from both male and female koalas that ranged from one to approximately 14 years of age. Each tissue sample consisted of a 2 mm in diameter plug of tissue created when the animals were ear tagged. Animals were captured by ‘flagging’ down from trees, and then restrained for a brief period by experienced koala handlers while ear tags were applied and tissue samples obtained. All work was authorised under licence (licence number: S10293) from New South Wales National Parks and Wildlife Service and ethics approval by

the relevant University bodies. A similarly sized piece of ear tissue was collected by sharp dissection from deceased animals. The tissue samples were stored in 70% ethanol at room temperature prior to DNA extraction. Tissues were processed with DNeasy tissue kits (QIAGEN) to extract genomic DNA following the DNeasy tissue kit protocol.

Repeatability and polymorphism levels of 17 microsatellite primers that had been previously identified (Houlden *et al.* 1996a; Cristescu *et al.* 2009) were tested in replicate PCR reactions with 12 randomly selected DNA samples from across the sample sites. Based on the ease of genotype scoring, error rates, reliability of PCR amplification, and level of polymorphism, 12 primer pairs were selected (results not shown). The 12 primer pairs used in analysis were: Phc 2, Phc 4, Phc 13 (Houlden *et al.* 1996a) and Pcv 2, Pcv 6.3, Pcv 24.2, Pcv 25.2, Pcv 26, Pcv 30, Pcv 31, K 2.1, K 10.1 (Cristescu *et al.* 2009).

DNA was amplified using PCR methodology based on M13 tailed forward primers (Neilan *et al.* 1997). Optimised conditions for PCR consisted of ~100ng of total genomic DNA, 10x PCR buffer, 0.5 mM each dNTP, 1.5 units *Taq* DNA polymerase (QIAGEN), $MgCl_2$ concentration of 1.5 mM, 0.25 uM of forward primer, 1 uM reverse primer, 0.5 uM of either NED, VIC, FAM or PET fluorescently labeled M13 primer (Applied Biosystems), and sterile water to bring to 10 ul total volume. Loci were amplified using a touchdown PCR protocol: initial 94°C denaturation for 3 minutes, followed by six touchdown cycles of 94°C for 30 seconds, annealing temperature at 60°C (and decreasing by 2°C each cycle) for 45 seconds, and extension step of 72°C for 60 seconds. On completion of the final touchdown cycle, a further 30 cycles were performed at the 50°C annealing temperature, followed by a final extension of 72°C for 10 minutes.

Amplification products for each sample were genotyped using an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Genotypes were scored using GeneMapper 4.0 (Applied Biosystems) and verified manually. All allele scorings were independently checked by eye by at least two people. All genotypes with low signal intensity or patterns that were difficult to interpret on GeneMapper 4.0 were re-electrophoresed and/or re-amplified. As a quality control measure, 30 random individuals were amplified a second time across all loci and re-scored blindly in order to assess error rates for each locus.

Loci and population statistics

Deviations from Hardy-Weinberg (HW) equilibrium and presence of null alleles and/or sub-structuring were assessed by measuring F_{IS} and its statistical significance (10,000 permutations) for all loci within all sampling regions using FSTAT 2.9.3 (Goudet 1995). In addition, Mendelian inheritance was confirmed where mother-offspring relationships were known. Genotypic linkage disequilibrium for each pair of loci was calculated in FSTAT 2.9.3(Goudet 1995). Number of alleles for each locus and average number of alleles across loci and populations was calculated in FSTAT 2.9.3 (Goudet 1995). The false discovery rate (FDR) control (Benjamini and Yekutieli 2001) for multiple testing was used where applicable for the p-values throughout this paper.

Population structure

Genotypic population structure was analysed using two programs, GENELAND 3.1.4 (Guillot *et al.* 2005a; Guillot *et al.* 2005b; Guillot 2008; Guillot *et al.* 2008) and STRUCTURE 2.1 (Pritchard *et al.* 2000; Falush *et al.* 2003). Both programs are based on Bayesian approaches that allow populations to be defined by the genetic data, rather than requiring an *a priori* estimation of population definition. In addition, GENELAND incorporates spatial information (the location in which each individual was sampled), and assigns greater probability to genetic clusters that are continuous within the spatial landscape (Guillot *et al.* 2005a).

GENELAND analysis was performed with an initial series of runs (12 runs at 500 000 Markov chain Monte-Carlo (MCMC) iterations each) to determine the most probable number of genetically distinct clusters (K). The uncertainty associated with the spatial coordinates was set to 1000m, to allow variation within the koala's home range (Ward 2002). Minimum K was fixed at one and maximum K at 12. The Dirichlet model was used as a model for allelic frequencies, and the option to take account for the presence of null alleles was selected (Guillot *et al.* 2008). Default values were used for remaining parameters. The number of populations was inferred based on the mode of these 12 runs. To assign individuals to the inferred number of populations, the MCMC was run 10 times with K set to the inferred number of populations (Guillot *et al.* 2005a). Other parameters remained the same as used in the runs with variable K. GENELAND was used to produce a Voronoi tessellation (Dupanloup *et al.* 2002) map of posterior probabilities of population membership for each of these 10 population assignment runs. To assess potential barriers to gene flow, a thematic map (1:200 000) including the potential barriers (eg. roads, waterways, land use) was compared visually with the tessellation map.

STRUCTURE analysis was performed with five runs at each value of K, with values of K set from one to 10. Maximum number of populations was set at 10 to give a large margin of error in our estimates of maximum number of populations, and to allow for possible genetic structuring within each site. Each run was performed with a burn-in of 50000 MCMC iterations, followed by 1 000 000 MCMC iterations. The correlated allele frequency model and the admixture model were used, because each sampling region may have some contact. All other values were set to their default values. The mean log likelihood of each K and the ΔK method described by Evanno et al. (2005) was used to estimate K. To assign individuals to populations we performed five final runs at the estimated K.

In order to visualise the genetic similarity between individuals and populations, a neighbour-joining (NJ) distance tree was constructed based on 1 – proportion of shared alleles between all individuals (Goldstein *et al.* 1999). The distance matrixes were generated using the program MICROSAT (Minch *et al.* 1995) and the NJ tree was built with the program MEGA version 2.1 (Kumar *et al.* 2001).

Population pairwise F_{ST} values were used to measure the level of genetic differentiation between the populations inferred by GENELAND and STRUCTURE. F_{ST} values and their significance (10 000 permutations)

were estimated in FSTAT 2.9.3 (Goudet 1995). For comparison, the same tests were then performed using each of our four original sampling regions as separate populations. To gain some indication of the timescale of the population splits and to assess whether the populations have been historically separated over the very long term, we compared observed R_{ST} values to R_{ST} values generated by random permutation of allele size information in the program SPAGEDI 1.3 (Hardy and Vekemans 2002). Significantly smaller permuted (pR_{ST}) than observed R_{ST} values suggest that mutation may have contributed to the observed population differentiation rather than genetic drift alone.

To test for spatial genetic structure at the level of individuals, a spatial autocorrelation test was performed in GENALEX 6 (Peakall and Smouse 2006), using the method of Smouse and Peakall (1999). The spatial autocorrelation coefficient was calculated for all genotypes and represented as a correlogram. Geographical distance was measured as linear Euclidean distance. To test for no spatial genetic structure in the combined data set, 95% confidence intervals were estimated using 999 permutations, while 999 bootstraps were used to estimated confidence intervals for *r* for given geographical distance classes.

Population genetic diversity

Genetic diversity within each of the three inferred populations was evaluated by calculating: mean number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), number of rare (frequency of less than 5 %) alleles (A_{RARE}) and number of unique alleles (A_U) using GENALEX 6.1 (Peakall and Smouse 2006). Allelic Richness (A_R), a measure that adjusts the alleles per locus to account for variation in sample size, was calculated in FSTAT 2.9.3 (Goudet 1995). Private allelic richness (A_{UR}), a measure that uses rarefaction to adjust the number of unique alleles per locus to account for variation in sample size was calculated in HPRARE 1.1 (Kalinowski 2004; Kalinowski 2005).

Testing for a recent population bottleneck

A combination of methods was used to test for evidence of a population bottleneck. The program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996) was used with the Two-Phase Mutation (TPM) (Di Rienzo *et al.* 1994; Luikart *et al.* 1998) model with 90% single step mutations (12% variance). Significance was assessed using Wilcoxon's sign-rank tests. In addition, the effective population size was estimated using ONESAMP 1.1 (Tallmon *et al.* 2008) which uses a Bayesian framework, and NEESTIMATOR 1.3 (Peel *et al.* 2004) which incorporates a linkage disequilibrium analysis. For these bottleneck and effective population size analyses, only the inferred Campbelltown population (i.e. Campbelltown and Heathcote sampling areas combined) was tested due to insufficient sample sizes in the other populations.

Results

Loci and population statistics

When evaluated across all populations, all loci were in Hardy-Weinberg equilibrium in the pooled sampling regions test. F_{IS} values for each population were not significantly different from zero. There was no significant linkage disequilibrium detected between any of the loci. All loci were polymorphic, and number of alleles per locus ranged from four (Pcv 2) to 13 (Pcv 6.3), with a mean of 7.33 alleles per locus. Blind re-scoring of genotypes of 30 animals did not result in any contradictions.

Population structure

Both GENELAND and STRUCTURE inferred that the most likely number of populations was three. The population boundaries found by GENELAND were well defined and geographically distinct (Figure 3) and corresponded to sampling region, except the Heathcote and Campbelltown animals that were consistently assigned to the same population. Similarly, STRUCTURE's assignments corresponded to the sampling regions and also grouped the Campbelltown and Heathcote animals together as one population, and all assignments were very strong ($q>0.9$). For all subsequent population analyses, the Campbelltown and Heathcote animals are to be considered a single population. The location of environmental barriers corresponded to GENELAND's border between the Campbelltown region and the Southern Tablelands population. The existence of three discrete populations was also clearly shown in the NJ distance tree (Figure 4) where three clear groups were identified, and these groups are virtually identical to the population assignment given by GENELAND and STRUCTURE. Spatial autocorrelation tests revealed no significant genetic spatial structure (ie., isolation by distance). The R_{ST}/pR_{ST} comparison test was not significant, indicating it is not possible to say the barriers are based on evolutionary timescales, and therefore it is possible that any observed differentiation is due to contemporary barrier to gene flow.

Genetic differentiation of inferred populations and of sampling regions

Pairwise F_{ST} of the inferred populations showed considerable differentiation, and all F_{ST} values were significant ($p<0.001$). Pairwise F_{ST} values were: $\theta = 0.224$ (Campbelltown/Heathcote – Southern Tablelands), $\theta = 0.220$ (Campbelltown/Heathcote – Blue Mountains) and $\theta = 0.141$ (Southern Tablelands – Blue Mountains). F_{ST} analysis was also performed based on sampling regions, and results were similar except the pairwise F_{ST} between Campbelltown and Heathcote was $\theta = 0.006$ ($p=0.297$).

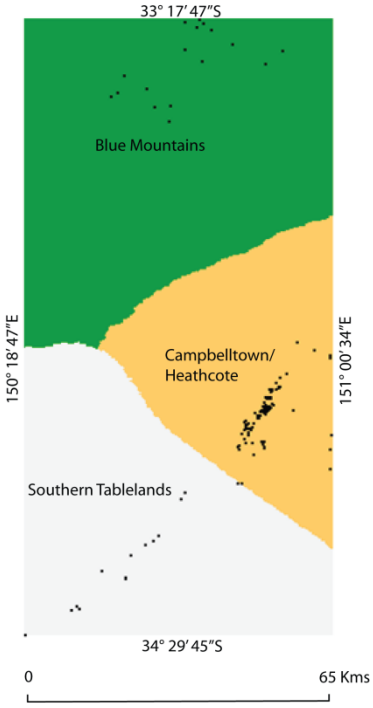

Figure 3 Individual assignments from GENELAND. Each sample is represented by a black dot and placed according to sample site spatial coordinates. Samples are grouped into one of three populations.

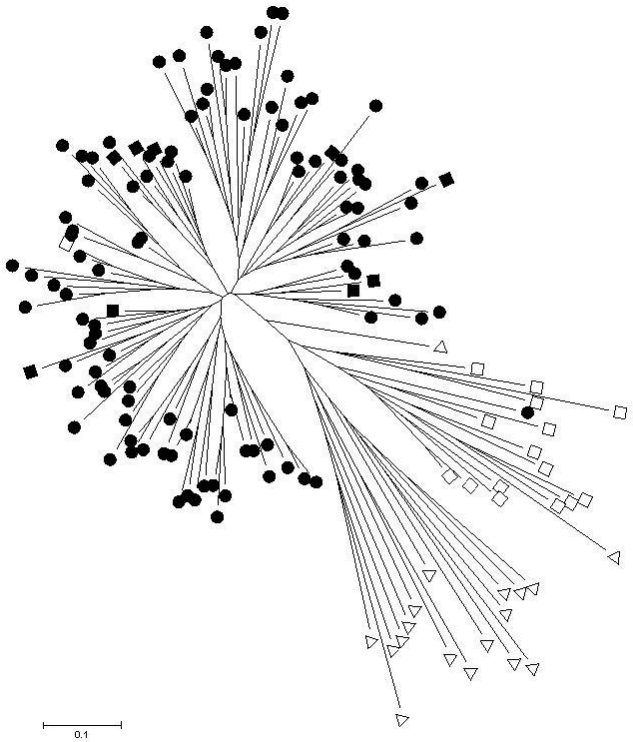

Figure 4 Unrooted neighbour-joining tree displaying 1- proportion of shared alleles genetic distance between sampled individuals. ● = Campbelltown, ■ = Heathcote, □ = Southern Tablelands, Δ = Blue Mountains.

Within-population genetic diversity

The Blue Mountains region had the highest genetic diversity and greatest proportion of unique alleles, while the Campbelltown/Heathcote population had the least genetic diversity and the fewest unique alleles. Observed (H_O) and expected (H_E) heterozygosity, average number of alleles per locus (A), allelic richness (A_R) and private allelic richness (A_{UR}) is provided in Table 1.

Testing for a recent population bottleneck

Analysis of the inferred Campbelltown/Heathcote population with BOTTLENECK detected significant evidence of a recent population bottleneck using the heterozygote excess test (p = 0.02). Effective population size estimates for this population using ONESAMP and NEESTIMATOR was very low and ranged from 16.3 (95% CI: 15.4, 17.3) to 21.2 (95% CI: 16.1, 29.7) respectively. The mode-shift test in BOTTLENECK did not detect evidence of a bottleneck. However, this may simply mean the bottleneck was of short duration, the bottleneck occurred sufficiently long ago that the population size has since recovered, or even that there has been a low level of migration, since any of these factors could prevent a mode-shift signal (e.g. Keller *et al.* 2001; Eldridge *et al.* 2004; Busch *et al.* 2007)

Table 1 Summary of genetic diversity parameters. [a]Total number of koalas genotyped. [b]All FIS values were not significantly different from zero.

	N[a]	A	A_R	A_{UR}	H_O	H_E	F_{IS}[b]
Campbelltown and Heathcote	110	3.17	2.93	0.11	0.499	0.542	0.079
Sth. Tablelands	16	5.08	4.88	0.88	0.520	0.586	0.113
Blue Mountains	18	6.83	6.46	2.62	0.655	0.743	0.118
Mean	-	5.027	4.757	15.333	0.558	0.624	-

Discussion

The impacts of external threats and viability of koala populations vary across their geographic range. Therefore, koala management plans in Australia need to be developed and implemented on a local scale. Although the NSW Koala Recovery Plan has identified the Campbelltown population as a priority population for study, there are no management plans specific to koalas in the Sydney region (DECC 2008). The objectives of this research were to formulate management recommendations that will maximise the long term conservation of koala populations in the Sydney region by resolving their genetic structure.

Campbelltown and Heathcote regions

The Campbelltown and Heathcote koalas should be considered a single continuous population. Both genic and genotypic analysis could not discriminate between the two sampling regions.

These sample regions are separated by the Holsworthy Army Range. This study shows that animals separated by the Army Range represent a single population. One can therefore assume that koalas use the range as important connective habitat for dispersal between the Campbelltown and Heathcote regions. Based on habitat considerations mentioned previously it appears likely, although cannot be proven from this data, that koalas also permanently inhabit the Army Range in addition to using it as an important thoroughfare.

While these genetically informed conclusions are based on only a relatively small sample size from the Heathcote region, there is also ecological evidence to further support the findings. Previously published estimates of male migration distance in other regions also confirm the potential for migration between the Campbelltown and Heathcote regions (e.g. Mitchell and Martin 1990; Dique *et al.* 2003)

There is evidence that the Campbelltown/Heathcote population has undergone a genetic population bottleneck. The BOTTLENECK analysis detected significant evidence of a population bottleneck when using the heterozygosity excess test (Cornuet and Luikart 1996). This is also supported by the small effective population size estimates for this region (16 and 21), especially considering the population census size is conservatively estimated to be at least 400 (Ward 2002). Finally, the reduced number of rare alleles and overall lower genetic diversity compared to the other populations, further supports the hypothesis of a sustained reduction in effective population size (Nei *et al.* 1975; Maruyama and Fuerst 1985). Earlier ecological data suggested a bottleneck was possible, since a severe disease outbreak occurred in the region in the early 1920s (Tilley and Uebel 1990) and koalas were not seen around Campbelltown from about 1920 until 1986 (Ward and Close 2004). The genetic data in this paper now support a bottleneck hypothesis.

Considering the low genetic diversity and strong evidence for a single continuous population, management of the Campbelltown and Heathcote regions as a single population incorporating the Holsworthy Army Range is essential and will minimise chances for the additional loss of genetic diversity. Relationships between neutral genetic marker diversity and functional genetic diversity are not always strong and a more accurate picture of genetic diversity would be obtained by directly measuring quantitative genetic traits (Reed and Frankham 2001). If the land use changes and koalas are no longer able to move through the Army range, this will cause a major fragmentation of the Campbelltown/Heathcote population and is likely to accelerate genetic drift and potentially lead to inbreeding. The future of the Holsworthy Army Range is currently uncertain. If the Army Range land is modified or developed in any manner, it will be essential that development plans incorporate strict measures to ensure gene flow is maintained across the range between the Campbelltown and Heathcote regions. Such measures are likely to include substantial migration corridors. Based on our evidence of barriers to gene flow in the south of the region, care would need to be taken to ensure the corridors were well clear of roads or housing and contain preferred koala habitat.

Population differentiation and barriers to gene flow

The Campbelltown/Heathcote, Southern Tablelands and Blue Mountains populations were clearly differentiated by each of the different methods of analysis in this study and therefore should be considered discrete populations. These three populations have apparently little gene flow between them. Isolation by distance was not detected, so the observed differentiation may be explained by the presence of barriers to gene flow and founder/bottleneck effects (Schwartz and McKelvey 2009).

Significant geographical and artificial barriers are present throughout our study area. Based on our genetic analysis and sampling locations, an abrupt demarcation was found between koalas in the Campbelltown/Heathcote population and the Southern Tablelands population. The R_{ST} analysis found no significant difference in observed versus permuted R_{ST} values, lending some support to the idea that these are contemporary barriers to migration rather than a substantially 'pre-European' evolutionary separation. Some samples were obtained from roadkill, which may have lead to non-random sampling in some areas. However, only a small minority of samples are roadkill, and the main bias introduced is likely to be towards young dispersing male koalas. If anything, this bias is likely to increase the ability to detect gene flow and therefore should not underestimate the amount of gene flow existing.

The low genetic diversity and small effective population size of the Campbelltown/Heathcote population could make it more vulnerable to habitat change and survival pressures in the future (Frankham *et al.* 2002). Allowing natural gene flow between the Campbelltown region and the Southern Tablelands population to occur would naturally enrich the genetic diversity in both populations by introducing new alleles, would increase overall N_e and reduce future loss of genetic diversity. (e.g. Frankham *et al.* 2002). Natural migration can be encouraged by creating and maintaining habitat corridors that allow the safe navigation through the potential barriers to gene flow mentioned above.

Key strategies for encouraging natural migration should also involve measures allowing koalas to safely cross the road separating the Campbelltown/Heathcote and Southern Tablelands populations. Our genetic results suggest few koalas safely cross this road, resulting in little effective gene flow. Creating road culverts (Taylor and Goldingay 2003) and/or reducing the speed limit (Dique *et al.* 2003) around koala blackspot zones may help reduce koala fatalities and facilitate safe road crossings.

While there was strong differentiation, it is possible a low level of individual migration is occurring, although not enough to create effective gene flow among generations. For example, the NJ tree (Figure 4) shows two potential candidates for migratory exchange between the Campbelltown and Southern Tablelands populations. Of these, the candidate migrant into the Campbelltown region in particular could be a genuine migrant, based on the fact that STRUCTURE also assigned this animal into the Campbelltown population. The

potential migrant into the Southern Tablelands as suggested by the NJ tree was not supported by STRUCTURE results.

Comparative genetic diversity

The Blue Mountains population had the highest level of genetic diversity and is comparable to the highest diversity found in Houlden et al.'s (1996b) study. Three of Houlden's six microsatellite primers were used in this study, allowing some degree of comparison between the studies. The Southern Tablelands population and the Campbelltown/Heathcote population however have much less diversity when compared to the Blue Mountains population. As we have no prior samples we cannot be sure if the Southern Tablelands population has 'lost' genetic diversity or has always had less than the Blue Mountains. However in Campbelltown, the BOTTLENECK program gives us some confidence that there has been a genuine loss of genetic diversity. Habitat in the Campbelltown/Heathcote and Southern Tablelands regions is generally considered high quality for koalas (Tilley and Uebel 1990; Ward 2002), so it is unlikely that the habitat historically supported smaller, less diverse populations. In addition, anecdotal evidence regarding the size of the koala fur trade in the Campbelltown region suggests the population was historically considerably larger than today. The lower genetic diversity seen in the Campbelltown/Heathcote and the Southern Tablelands populations is possibly the result of the fur trade, disease (Tilley and Uebel 1990) or habitat loss. Relationships between neutral genetic marker diversity and functional genetic diversity are not always strong and a more accurate picture of genetic diversity would be obtained by directly measuring quantitative genetic traits (Reed and Frankham 2001). The Blue Mountains koalas may have been spared some of the impact of the fur trade or habitat loss because they inhabit terrain that is more remote and difficult to access. The Blue Mountains population appears to be of high conservation value as it holds a reservoir of genetic diversity not seen in other populations in the Sydney region. Far fewer animals were sampled in the Blue Mountains and the Southern Tablelands populations than in the Campbelltown/Heathcote population and therefore an increased sample size may uncover even more genetic diversity for these populations.

Areas for future research

Few recent sightings of koalas in the forests to the southeast of the Campbelltown region have been recorded, possibly because these areas are part of the Sydney water catchment and access is restricted. Surveys for koalas and analysis of genetic material from known populations on either side of the disputed region may help to determine if animals are present in this area. The major highway between Sydney and Canberra bisects the Southern Tablelands population. Most of our public sightings and capture samples from came from animals on the western side of this highway. Additional samples from the eastern side of the highway could be used to test the hypothesis that large roads such as this pose a barrier to gene flow, assuming the roads have created a new barrier and were not built on landscape features that already had a barrier effect. Our analyses of Sydney koalas may provide insights into factors that may affect other mammals in the region, as they have shown that while some mammals can exist in highly fragmented semi-urban areas, certain landscape features are able to further reduce gene flow. Therefore research into other species in the outer Sydney region should consider

landscape features as potentially causing further fragmentation in populations already fragmented through loss of habitat.

Conclusion

This paper has identified three discrete koala populations in the Sydney region. Little gene flow among these populations was inferred. Although the Campbelltown/Heathcote and Southern Tablelands populations abut, there appears to be a barrier to gene flow between them that may be the result of geographic features, human alterations of the land or a combination of these. Also revealed is the high level of genetic diversity in the Blue Mountains population, which is comparable to the highest levels previously published for koala populations. However, the Campbelltown/Heathcote population has relatively low diversity, and there is evidence indicating this population has suffered a recent genetic bottleneck. The fact that these are demographically separate populations has important implications for koala management in the Sydney region. The three confirmed koala populations should be considered separate management units and will need specific management plans tailored to the conservation issues and priorities for those regions. This paper has also shown that the army land is likely to be critical for the viability of the Campbelltown population because of the connectivity it provides for the Campbelltown and Heathcote regions. The most effective and the simplest solution for conservation of koalas in the Sydney region would appear to be preventing or limiting any further loss of population connectivity.

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Genetic analysis reveals a distinct and highly diverse koala (*Phascolarctos cinereus*) population in South Gippsland, Victoria, Australia.

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Abstract

Population genetics can reveal otherwise hidden information involving a species' history in a given region. Koalas were thought to have been virtually exterminated from the Australian state of Victoria during the koala fur trade of the late 1800's. Koalas in the South Gippsland region of Victoria, Australia, were examined using microsatellite markers to infer population structure and gene flow and to locate a possible remnant gene pool. The results indicate that the South Gippsland koala population had higher genetic diversity (A=5.97, H_O=0.564) than other published Victorian populations, and was genetically distinct from other koala populations examined. South Gippsland koalas, therefore, may have survived the population reductions of the koala fur trade and now represent a remnant Victorian gene pool that has been largely lost from the remainder of Victoria. This paper illustrates that historic anthropogenic impacts have had little effect on reducing the genetic diversity of a population in the South Gippsland region. However, the South Gippsland population is now subject to threats such as logging and loss of habitat from housing and agriculture expansion. Our results suggest the South Gippsland koalas require an alternative conservation management program.

Introduction

Koalas are iconic Australian marsupials that have experienced a turbulent history. Although koalas are widespread across the eastern coast of Australia (Figure 5), many populations have been impacted by localised extinctions, population bottlenecks, reintroductions and overpopulation, all of which can affect the health and viability of these animals. Population bottlenecks may reduce genetic diversity which limits the ability of the

population to adapt to change, while mismanagement of some koala populations has resulted in overpopulations and consequently over-browsing to the point where severe defoliation has occurred, resulting in starvation (Cristescu *et al.* 2009; Lee *et al.* 2010; Masters *et al.* 2004). Houlden *et al*. (1996b) have shown that Victorian koalas have significantly lower levels of genetic diversity compared to other koala populations in Australia, which have been linked to a population crash caused by the koala fur trade and subsequent reintroductions from island populations with limited gene pools. Koala hunting and fur export occurred over a period of up to 30 years in some parts of Australia (Gordon and Hrdina 2005; Serventy and Serventy 1989), from the late 1800's to early 1900's. Between 1919 and 1924 eight million koalas were killed and their pelts exported (Martin and Handasyde 1999). The koala fur trade occurred across eastern Australia and resulted in rapid population declines and extinctions in many areas of the koala's original range.

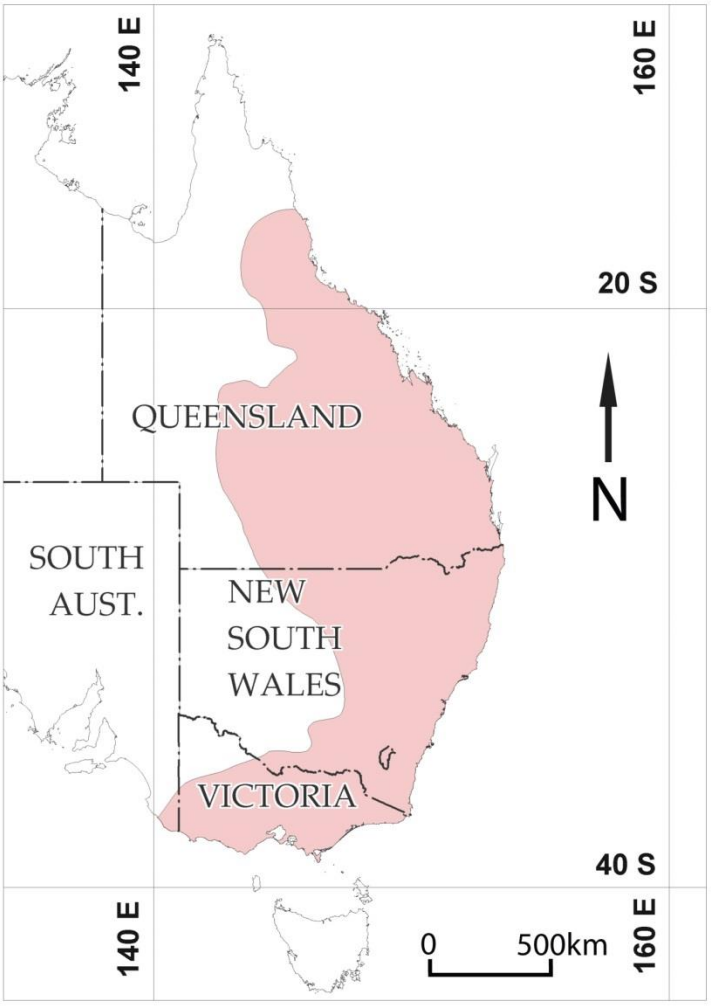

Figure 5 East coast map of Australia with shading indicating the broad geographical range of koalas. Koala populations in this range are highly fragmented. Reproduced with permission from the Australian Koala Foundation

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Koala populations in the Australian state of Victoria were so greatly reduced by the fur trade that a small number of koalas were moved to French Island off the coast of Victoria in an effort to save them from extinction (Martin and Handasyde 1999). Anecdotal evidence suggests the French island population may have been established with as few as two or three individuals (Martin and Handasyde 1999). Furthermore, genetic analysis has revealed severely reduced genetic diversity of French Island animals with only two mitochondrial DNA haplotypes discovered (Taylor *et al.* 1997). Following the end of the fur trade around the late 1920's, koalas were gradually restocked onto the mainland of Victoria through a translocation program, using animals from the bottlenecked French island population (reviewed by Houlden *et al.* 1996b). Although successful in increasing numbers of koalas on the mainland, the program has resulted in koala populations in Victoria possessing lower genetic diversity compared to koala populations in the rest of Australia (Houlden *et al.* 1996b). Victorian populations derived from translocated animals have an average reported allelic diversity of around 3 alleles/locus (Houlden *et al.* 1996b) compared to populations in NSW which have up to 6.83 alleles/locus (Lee *et al.* 2010). However, the low genetic diversity of some southern populations has not impeded their ability to successfully recolonise their former range, and koalas in many areas of Victoria and South Australia are now considered to be overabundant (Duka and Masters 2005; Masters *et al.* 2004). Indeed, the contemporary translocation program is now aimed at managing the problems of overpopulation on some islands.

The area of South Gippsland, in particular the Strzelecki Ranges (see Figure 6), in southeast Victoria has received only minimal numbers of translocated island animals (DSE 2004). It is thought that a small population in this region may have survived the fur trade, and therefore may retain remnant genetic diversity present in Victorian koalas prior to the fur trade. While results from studies using mitochondrial DNA are consistent with this hypothesis, those from previous microsatellite studies are not. Taylor *et al.* (1997) and Houlden *et al.* (1999) using mitochondrial DNA found a significant difference in haplotype frequencies between the South Gippsland population and populations involved in the translocation program. However, Houlden *et al.* (1996b) and Seymour *et al.* (2001) did not detect significant differences between French Island and South Gippsland koala populations when investigating microsatellite loci.

The Victorian State Government has recognised the potential importance of the South Gippsland koala population. The Victorian Koala Management Strategy (DSE 2004), lists "Managing Genetic Structure" as a key issue with a focus on possible remnant genetic resources persisting in South Gippsland koalas. The report states that a high priority point of action is to conduct a "detailed survey of genetic diversity" across the South Gippsland region. The Victorian State Government currently lists koalas in Victoria as "not threatened". Koalas are currently not listed under the Australian Government's endangered species legislation as either endangered or vulnerable, although this status is currently under review.

The main aim of this study is to use genetic techniques to investigate fine scale population structuring of koalas in the South Gippsland region. Information gathered will assist in establishing if a remnant Victorian

koala gene pool still exists. In addition, the Mornington Peninsula koala population is the nearest mainland population to French Island and has a documented history of receiving regular translocations from French Island. These mainland koalas could reasonably be expected to share genes with French Island koalas, and have been included in this study to investigate if translocation programs have had an impact on the Mornington Peninsula population. The Mornington Peninsula population was also included to assist in determining the geographical extent of any remnant Victorian koala gene pool.

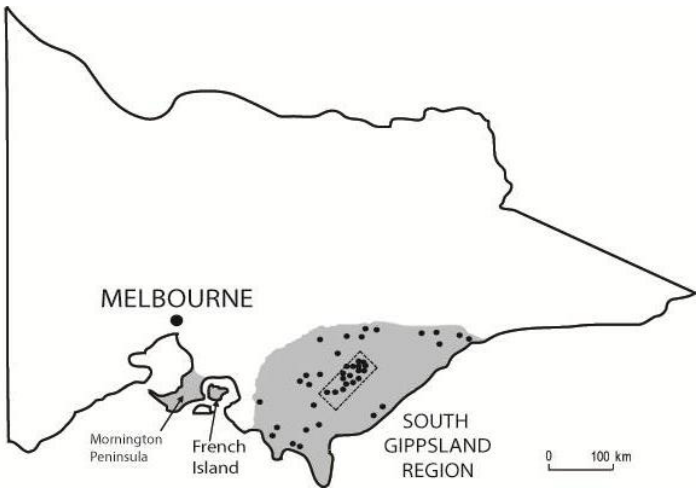

Figure 6 The state of Victoria showing sampling regions (shaded) and location of previous koala studies in the region. The location of the Strzelecki ranges is shown here by the dotted line border

Materials and Methods

Study area

Samples or genetic data were obtained from three geographic regions: South Gippsland (n=94), Mornington Peninsula (n=11) and French Island (n=49) for a total of 154 animals. The South Gippsland sampling area shown in Figure 6 corresponds to the area discussed in the Victorian Government report (DSE 2004). Tissue samples were obtained throughout the entire shaded area in Figure 6, with a particular emphasis on the Strzelecki ranges. French Island koala data was included in this study to determine if the South Gippsland koalas are derived from French Island translocations. Mornington Peninsula koalas were sampled to establish if they are derived from French Island translocations. Koala samples from South Gippsland and Mornington Peninsula were collected during the period of 2008 to 2009 as part of this study. French Island genetic data was obtained from a previous study by Cristescu *et al.* (2009) which was collected during 2002-2004. Koalas from all three locations inhabit eucalyptus forests, covering terrain varying from rugged, steep, mountainous areas to undulating tablelands with scattered clearing for agriculture. The South Gippsland and Mornington Peninsula samples are separated by an extensive area of agricultural land.

Sample collection and genotyping

South Gippsland and Mornington Peninsula samples were obtained opportunistically from deceased animals along roadways and from animals admitted to veterinary hospitals in the region, particularly as a result of widespread bushfires in 2009. Tissue samples were collected from both male and female koalas, ranging in age from one to approximately 10 years. Each sample consisted of an approximately 5cm x 5cm piece of ear tissue collected by sharp dissection. Work was authorised under Scientific Permit Number 10005270 from Victorian DSE. The tissue samples were stored in 70% ethanol at room temperature prior to DNA extraction. DNA was extracted from tissues using DNeasy tissue kits (QIAGEN) following the DNeasy tissue kit protocol.

Repeatability and polymorphism levels of 12 microsatellite primers that had been previously identified (Cristescu *et al.* 2009; Houlden *et al.* 1996a) were tested in replicate PCR reactions with 12 randomly selected DNA samples from across the available sample sites. Based on the ease of genotype scoring, error rates, reliability of PCR amplification, and level of polymorphism, nine primer pairs were selected (results not shown). The nine primer pairs used in the genetic analysis across all populations were: Phc 2, Phc 4, Phc 13 (Houlden *et al.* 1996a) and Pcv 2, Pcv 6.3, Pcv 24.2, Pcv 30, Pcv 31, K 10.1 (Cristescu *et al.* 2009). Individual genotypic data for the French Island individuals was obtained from Cristescu *et al.* (2009). To standardize French Island genotype data with the new data from this study, several representative animals was kindly provided by Romane Cristescu and included in the current genotyping analysis.

Microsatellite loci were amplified following Lee *et al.* (2010) and amplification products for each sample were genotyped and resolved using an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Microsatellite genotypes were scored using GeneMapper 4.0 (Applied Biosystems) and verified manually by at least two people. Genotypes with low signal intensity or patterns that were difficult to interpret on GeneMapper 4.0 were re-electrophoresed and/or re-amplified. As a quality control measure, eight random individuals were amplified a second time across all loci and re-scored blindly in order to assess error rates for each locus.

Loci and population statistics

Deviations from population Hardy-Weinberg (HW) equilibrium and the presence of null alleles and/or sub-structuring were assessed by measuring F_{IS} and its statistical significance (10,000 permutations) for all loci within all sampling regions using FSTAT 2.9.3 (Goudet 1995). Genotypic linkage disequilibrium for each pair of loci was calculated in FSTAT 2.9.3 (Goudet 1995).

Population structure

Genotypic population structure across all populations was analysed using STRUCTURE 2.3.3 (Falush *et al.* 2003; Pritchard *et al.* 2000). In addition, GENELAND 3.1.4 (Guillot 2008; Guillot *et al.* 2005a; Guillot *et al.* 2005b; Guillot *et al.* 2008) was used to test for possible substructuring in the South Gippsland sampling region as it is a very large geographical area. Both programs are based on Bayesian approaches which allow populations to

be defined by the genetic data, rather than requiring an *a priori* estimation of population definition. In addition, GENELAND incorporates spatial information (the location in which each individual was sampled), and assigns greater probability to genetic clusters that are continuous within the spatial landscape (Guillot *et al.* 2005a).

STRUCTURE analysis was performed with five runs at each value of K, with values of K set from one to eight. Maximum number of populations was set at eight to give a large margin of error in our estimates of maximum number of populations. Each run was performed with a burn-in of 50000 MCMC iterations, followed by 1 000 000 MCMC iterations. The correlated allele frequency model and the admixture model were used, because each sampling region may have some contact. All other values were set to their default values. The mean log likelihood of each K and the ΔK method described by Evanno et al. (2005) was used to estimate K. To assign individuals to populations we performed five final runs at the estimated K.

GENELAND analysis was performed with an initial series of runs (five runs at 500 000 Markov chain Monte-Carlo (MCMC) iterations each) to determine the most probable number of genetically distinct clusters (K). The uncertainty associated with the spatial coordinates was set to 1000m, to allow for a degree of error in reported location of each sample. Minimum K was fixed at one and maximum K at eight. The Dirichlet model was used as a model for allelic frequencies, and the option to take account for the presence of null alleles was selected (Guillot *et al.* 2008). Default values were used for remaining parameters. The number of populations was inferred based on the mode of these five runs. To assign individuals to the inferred number of populations, the MCMC was run five times with K set to the inferred number of populations (Guillot *et al.* 2005a). Other parameters remained the same as used in the runs with variable K.

Population pairwise F_{ST} values were used to measure the level of genetic differentiation between the populations inferred by STRUCTURE. F_{ST} values and their significance (10 000 permutations) were estimated in FSTAT 2.9.3 (Goudet 1995). In addition, a neighbour-joining (NJ) distance tree was constructed to visualise the genetic similarity between individuals and populations,. Distance matrixes based on Smouse and Peakall (1999) were generated using the program GENALEX 6.1 (Peakall and Smouse 2006) and the NJ tree was built with the program MEGA version 2.1 (Kumar *et al.* 2001).

Population genetic diversity

Genetic diversity within each inferred population was evaluated by calculating: mean number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and number of unique alleles (A_U) using GENALEX 6.1 (Peakall and Smouse 2006). Allelic Richness (A_R), a measure that adjusts the alleles per locus to account for variation in sample size, was calculated in FSTAT 2.9.3 (Goudet 1995). Differences in the number of alleles per locus (A, A_U and A_R) and observed heterozygosity between populations was tested using the Wilcoxon signed-rank test.

Testing for a recent population bottleneck

To test for evidence of a population bottleneck in the South Gippsland and French Island populations the program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996) was used. The infinite allele model (IAM) and Two-Phase Mutation (TPM) (Di Rienzo *et al.* 1994; Luikart *et al.* 1998) model with 90% single step mutations (12% variance) were used. Significance was assessed using Wilcoxon signed-rank tests.

Results

Data integrity and population statistics

All populations were in HW equilibrium and F_{IS} values were not significantly different from zero. There was no significant linkage disequilibrium detected between any of the loci. All loci were polymorphic, and number of alleles per locus ranged from 4 to 13 (Pcv 2 and Pcv 6.3 respectively). None of the blind replicates produced any contradictions.

Population structure

Both genotypic and genic methods inferred that the most likely number of populations was two. The two populations inferred by STRUCTURE were well defined and corresponded to sampling region of South Gippsland and Mornington Peninsula + French Island. For subsequent genetic diversity analysis, the French Island and Mornington Peninsula populations were combined and can be considered a single population since they were genetically indistinguishable. Pairwise F_{ST} between South Gippsland and Mornington Peninsula + French Island showed strong differentiation ($\theta = 0.250$), and was statistically significant ($p<0.05$). GENELAND inferred the most likely number of populations within the South Gippsland sampling region was one. The existence of two discrete populations (South Gippsland and Mornington Peninsula + French Island) was also clearly shown in the NJ distance tree (Figure 7). The NJ genetic distance tree also confirmed that Mornington Peninsula animals were dispersed evenly amongst French Island animals with no apparent separation.

Table 2 Summary of genetic diversity parameters for discrete populations: Na Total number of koalas genotyped, A: Allelic Diversity (average number of alleles per locus), AR: Allelic richness (alleles per locus, corrected for sample size), HO: Observed heterozygosity, H_E: Expected heterozygosity, A_U: Unique alleles and F_{IS}: Inbreeding coefficient.

	N^a	A	A_R	H_O	H_E	A_U	F_{IS}
South Gippsland	94	6.44	5.97	0.564	0.621	31	0.07
Mornington Pen. +French Island	60	4.3	3.91	0.375	0.465	15	0.014
Mean	-	5.65	5.30	0.47	0.50		-

Within-population genetic diversity

Observed (H_O) and expected (H_E) heterozygosity, average number of alleles per locus (A) and allelic richness (A_R) is provided in Table 2. The South Gippsland population has a mean of 6.44 alleles per locus, compared to French Island + Mornington Peninsula with a mean of 4.3 alleles per locus. The mean number of alleles per locus was significantly different ($p<0.05$) when tested using a Wilcoxon signed-rank test. However, observed heterozygosity (South Gippsland H_o=0.564 and French Island+Mornington Peninsula H_o=0.375) was not significantly different ($p>0.05$) between the populations. Allelic richness for South Gippsland was A_R =5.97, compared to A_R = 3.91 for Mornington Peninsula + French Island. The South Gippsland population has 31 unique alleles and 24 rare alleles. The French Island + Mornington Peninsula population has 15 unique alleles and 8 rare alleles. The differences in the number of rare and unique alleles (between populations) were statistically significant ($p<0.05$) when testing using the Wilcoxon signed-rank test.

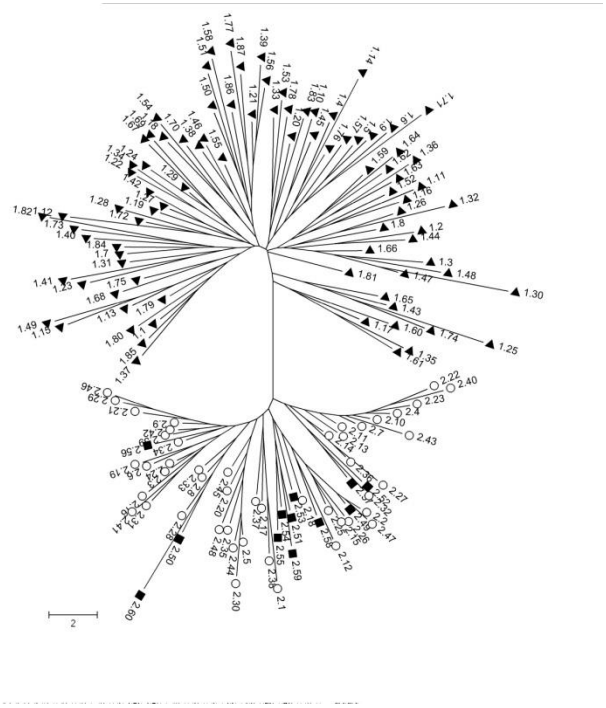

Figure 7 Unrooted neighbour-joining tree displaying genetic distance relationships between individuals (numbers refer to sample number) from South Gippsland (▲), French Island (○), and Mornington Peninsula (■).

Testing for a recent population bottleneck

Analysis of the South Gippsland population with BOTTLENECK did not detect evidence of a recent population bottleneck. French Island analysis detected significant evidence of a recent population bottleneck using the heterozygote excess test under the IAM (p = 0.002) but not under the TPM test. The mode-shift test for French Island in BOTTLENECK did not detect evidence of a bottleneck. However, this may simply mean that the bottleneck occurred sufficiently long ago with a rapid growth phase, causing the loss of a detectable bottleneck signal (e.g. Keller *et al*. 2001; Eldridge *et al*. 2004; Busch *et al*. 2007).

Discussion

South Gippsland region

Genic and genotypic analysis discriminated between the South Gippsland animals and nearby animals in the Mornington Peninsula and French Island, suggesting that the South Gippsland koalas should be considered a separate management unit to others in the region. Compared to previous Victorian koala research (Cristescu *et al.* 2009; Houlden *et al.* 1996b) the South Gippsland koalas stand out with their much higher genetic diversity. Combined with records showing limited translocations into the area, the South Gippsland population could be an endemic population not derived from reintroduced island populations.

Genetic data from this study indicate that the South Gippsland gene pool has not been homogenised with translocated island animals. Previous studies have presented differences between mitochondrial and microsatellite results. Taylor *et al.* (1997) and Houlden *et al.* (1999) using mitochondrial DNA found unique haplotypes while Houlden *et al.* (1996b) using microsatellite markers found no significant population differentiation between koala populations in South Gippsland and the rest of Victoria. We propose that a possible reason for the differences is the considerable difference in sampling locations. Our study used samples from throughout the entire area shaded in Figure 6, with a particular emphasis on the Strzelecki ranges. Approximately 40% of our South Gippsland samples come from the Strzelecki ranges, and these mountain ranges have no recorded history of koala translocations and have not been sampled previously. This compares with previous research which has used samples from the far south western edge (approx 80km from the Strzelecki Ranges) (Houlden *et al.* 1996b) and northern edge (Seymour *et al.* 2001) of our study area, both of which are also near recorded translocation sites (DSE 2004). As a verification measure, we repeated our analysis using only the three markers in common with Houlden *et al.* (1996b): the structure results were the same and F_{ST} results remained virtually unchanged. The increased number of markers does not alone account for the differences in the studies, and the likely explanation is a genuine population difference detected through the increased sampling efforts.

Comparative genetic diversity

The South Gippsland population had higher genetic diversity than French Island and Mornington Peninsula animals. The level of genetic diversity found in the South Gippsland population is the highest reported in Victoria and is comparable with the highest levels of genetic diversity in any koala population reported so far in Australia (Houlden *et al.* 1996b; Lee *et al.* 2010). Three of the six microsatellite markers used in Houlden *et al.* (1996b), and nine of the 12 used in Lee *et al.* (2010) were used in this study allowing some degree of comparison between the studies. Cristescu *et al.* (2009) found an average of 3.8 alleles for French Island, which is slightly less but not unexpected to the French Island + Mornington Peninsula population (A=4.3) in this study. The South Gippsland population as defined in Houlden *et al.* (1996b) had a mean number of alleles of approximately 4.25 compared to 6.44 in this study. However, at least some of this difference might be explained by the considerably different sampling areas in the South Gippsland region. South Gippsland possibly

harbours some of the diversity present more widely in Victorian koalas before the fur trade and translocation program. While relationships between neutral genetic marker diversity and functional genetic diversity are not always strong (Reed and Frankham 2001), the South Gippsland population appears to be of relatively high conservation value as it holds a reservoir of genetic diversity not seen in other populations in Victoria. There is no evidence of a bottleneck in the South Gippsland population. It is possible the South Gippsland koalas inhabited a terrain that is more remote and difficult to access, particularly in the Strzelecki Ranges in the centre of our sampled South Gippsland region and thus were spared the hunting pressures that other Victorian populations experienced.

Management Considerations

Although the Victorian State Government has identified the South Gippsland population as a priority population for study, there are currently no management plans specific to koalas in the South Gippsland region. In the Victorian Government document "Victoria's koala management strategy" (DSE 2004) Objective 7 states: "conserve the remnant genotype in South Gippsland koalas", assuming a remnant genotype is found. This study indicates a possible remnant genotype, and the next phase of research should investigate the possibility more closely and attempt to assess the size and distribution of the South Gippsland population.

Most of the South Gippsland region has lost koala habitat to agriculture. Local anecdotal accounts indicate some of the best habitat in the South Gippsland region is in the Strzelecki Ranges, parts of which may be altered and impacted by logging. If logging in the Strzelecki Ranges causes further habitat fragmentation of the South Gippsland habitat it may isolate koala populations and accelerate genetic drift. It is essential that logging plans incorporate measures to maintain koala gene flow between populations in logging areas and only minimal habitat is removed. Such measures need to include substantial migration corridors. Previous studies indicate that a variety of landscape features can present barriers to koala gene flow in the Sydney region (Lee *et al.* 2010), and therefore the corridors will need to take into account the presence of roads or housing and contain preferred koala habitat.

The well documented translocations from French Island into the Mornington Peninsula appear to have homogenised gene frequencies with any population remaining in the Mornington Peninsula after the fur trade. Our genetic study shows the Mornington Peninsula and French Island koalas are genetically similar, and could be considered a single population. Therefore, if a site is needed for excess French Island animals, and there is a desire to avoid 'contaminating' other populations with French Island's depauperate gene pool, moving French Island animals onto the Mornington Peninsula is analogous to moving koalas around within their original population.

Supplementing Victorian populations using South Gippsland animals

Victoria's koala management strategy document (DSE 2004) proposes artificially disseminating the South Gippsland gene pool through existing Victorian populations. The low genetic diversity of other Victorian koalas

could make them more vulnerable to habitat and climate change and other survival pressures in the future (Frankham *et al.* 2002). Supplementing the limited gene pool with animals from South Gippsland may be beneficial but could only be undertaken provided there were no adverse effects on the South Gippsland population. Information such as population size and demographics would be essential in constructing a supplementation program that will not be detrimental to the South Gippsland population. If South Gippsland animals were to be translocated, further studies investigating possible local adaptation effects would be needed to ensure the source koalas are able to survive the new environments.

Victoria's koala management strategy also raises the possibility of introducing South Gippsland animals into currently unoccupied but apparently suitable habitat. However, a substantial number of founding animals would be needed to avoid the problems with small founding populations and bottlenecks as already seen on Victorian islands. Resources and effort should therefore be directed at maintaining viable habitat within the South Gippsland region, rather than moving koalas away to establish new populations and beginning the bottleneck/reintroduction cycle all over again.

Conclusion

This study suggests the existence of a distinct and diverse koala gene pool in the South Gippsland region of Victoria, Australia. The South Gippsland population has apparently suffered few of the effects of the translocation programs that have affected many other Victorian populations. This study has also confirmed the historical records by showing the Mornington Peninsula animals are derived from French Island animals to such an extent they can be considered one population. The high level of genetic diversity in the South Gippsland population is also revealed. The South Gippsland population should be considered a separate management unit and will need specific management plans tailored to the conservation issues and priorities for the region. The most effective and the simplest solution for conservation of koalas in the South Gippsland region would appear to be preventing or limiting any further loss of population connectivity, especially within parts of the relatively undisturbed Strzelecki Ranges.

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Conclusions

Koalas can be difficult animals to study as they are cryptic species and can occur at very low densities. As a result, our knowledge of koalas is surprisingly low, considering they are one of Australia's most well-known native animals. One way to fill the knowledge gap and learn about those aspects of koala's biology that cannot easily be observed is through genetic studies. For the two koala population studies in this thesis we used genetic methods to provide detailed knowledge of koala populations that would have otherwise been impossible to obtain through ecological observations alone. In Chapter Two, we used population genetics to complement and expand upon a research program based on koala sightings and radio-telemetry. Similarly, in Chapter Three we employed genetic methods to discover the history of a koala population and implications for its future.

We have shown in Chapter 2 that a koala population most likely exists within an Army Range at Holsworthy, NSW and this population provides a link between koalas in Campbelltown, NSW and those further east near Heathcote, NSW. The Campbelltown population should therefore be thought of as a larger population, including those Heathcote koalas. This conclusion has implications for management of the important Campbelltown population and the habitat provided by the Holsworthy Army Range. For example, the importance of the habitat within the Holsworthy Army Range in maintaining a healthy koala population should be considered when making decisions on the future uses of the Range. In addition, the Campbelltown and Heathcote populations should be considered together when devising management plans or assessing impacts.

In Chapter Two we suggest the existence of a barrier to gene flow between the Campbelltown population and the Southern Highlands population to the south. The barrier between populations corresponds with a significant roadway, leading us to conclude the road and associated infrastructure may be the barrier to gene flow. The immediate reaction is therefore to recommend measures to improve gene flow between populations, as a means of avoiding problems with low genetic diversity and genetic drift. However, opening up movement between the Campbelltown population to migration from the south could prove to be a disaster if such an action introduces Chlamydia to the Campbelltown population from the Southern Highlands population. Six koalas from the Southern Highlands population (as defined in Lee et al. (2010)) have been presented showing clinical signs of Chlamydia and were PCR positive for Chlamydia (David Phalen, unpubl. Data, 2013). Therefore the roads acting as a barrier to koala migration could be protecting the Campbelltown population from Chlamydia infection from further south, in which case, care should be taken before conducting any interventionist activities such as artificial translocation of animals. Scenarios such as this highlight the perils and complexity around decisions to intervene in wildlife management.

Chapter Two also reveals that the northern Blue Mountains population is a highly genetically diverse population. This could suggest the presence of a large and widespread population, although very little is known about the size and distribution of the Blue Mountains population. Habitat connectivity between the

Southern Highlands population and the southern edges of the Blue Mountains population means it is conceivable that there would be a degree of migration between the two populations. The same could be said for populations to the north of the Blue Mountains, such as Yengo National Park (Curtin et al. 2002). The implications of this study are therefore that the Blue Mountains population could be one part of a very large koala population roughly following the national parks and other protected areas along the Great Dividing Range.

Chapter Three contains a study in which we use population genetics to discover the history of the South Gippsland koala population. The South Gippsland population appears to have survived the fur trade and has not been mixed with subsequent mass translocations of island stock. We present evidence to suggest a widespread population with much higher genetic diversity than other Victorian koala populations. We therefore conclude that the South Gippsland population should be managed as a distinct management unit in Victoria. The history, significance and genetic diversity of the South Gippsland koalas means that priority should be given to their conservation by Victorian wildlife managers. Future research should focus on understanding the size, distribution and density of this population. There may even be potential to use the South Gippsland koalas to provide genetic supplementation to other Victorian population, although this would need to be conducted with the appropriate precautions and risk management, as outlined in Chapter Three.

Comprehensive knowledge of all aspects of individual koala populations will only become more important now that koalas are listed by the Australian Government as a threatened species. Fine-scale genetic studies are useful methods to understand populations, and as shown in this thesis can be powerful tools when used in conjunction with other methods. The Australian Government listing also means that decisions will need to be made more regularly and perhaps with greater implications than before. Major developments such as housing, mining or infrastructure will increasingly encroach on koala habitat. It is therefore vital that planners and governments have the best possible information to make the decisions that will necessarily be a balance of social, economic and environmental factors.

One area of future research could focus on using population genetics to identify discrete populations worthy of a higher conservation status and legal protection. This would require sampling of most or all known koala populations across Australia, followed by fine scale genetic analysis to delineate the boundaries of any populations identified as potentially having high genetic value. Of course, genetic studies will only be as good as the DNA samples that are used. One limitation to conducting wide-ranging studies is the difficulty in obtaining high-quality DNA. The highest quality DNA comes from samples from captured or recently deceased koalas. Fresh samples are difficult to obtain in remote and low density populations, due to the infrequency with which koalas are encountered. Faecal DNA collection can be a means of more easily obtaining a greater number of samples, although faecal DNA is generally of low quality making genetic analysis unreliable. For such a large project, presumably involving many researchers, consistency of analysis would be important and a standard set of markers should be used for all studies. For example, those from Cristescu et al. (2009) could be

suitable as a standard suite of microsatellite markers assuming microsatellites remain the markers of choice. When populations are identified and described by researchers, recommendations can be made to governments for the conservation and management of those koala populations. Effective koala conservation management for these specially identified populations would require improving habitat models (Cork et al. 2000), managing the distribution and demographics of populations (Melzer et al. 2000, Penn et al. 2000, Phillips 2000), and research into reproductive success (Sherwin et al. 2000). Tsangaras et al. (2012) suggest that mitochondrial DNA markers may have limited utility in future koala studies, as they conclude that low mitochondrial diversity cannot be interpreted to mean recent inbreeding or a bottleneck event. According to Tsangaras et al. (2012) koalas have exhibited similar low levels of genetic diversity dating back to the early 1800s, well before the koala fur trade.

The two studies in this thesis have implications for current koala management practices in NSW and Victoria. Under NSW Government legislation, the State Environment Planning Policy 44 (SEPP44) is a legal instrument that is intended to control development within koala habitat in certain listed NSW local government areas (LGAs). SEPP44 applies to two types of koala habitat: "potential koala habitat" and "core koala habitat". Potential koala habitat is determined based on tree type and core koala habitat is defined by SEPP44 as "land with a resident population of koalas…such as breeding females and recent sightings of and historical records of a population". Schedule 1 of SEPP44 provides for the listing of LGAs where SEPP44 applies. At the time of writing this thesis, SEPP44 applied to 108 LGAs, including Campbelltown, as well as Wollondilly and Wingecarribee in the Southern Highlands. The genetic evidence provided in Chapter Two strongly supports the addition of Sutherland Shire Council LGA in Schedule 1 of SEPP44 due to the evidence of koala population in the Heathcote and Holsworthy Army Range areas. Chapter Two provides substantial evidence that the Campbelltown population extends into the Sutherland Shire LGA. Ward (2002) reached a similar conclusion regarding Sutherland and provided similar recommendations based on their use of radio-tracking and community sightings data.

In future genetics studies of koalas, using similar markers would allow studies to be compared to each other's, as well as creating the possibility of sharing data between researchers to allow broader studies and comparisons between more populations. Studies incorporating samples from geographically diverse or distant locations are often impractical for a single researcher to conduct. If an agreed suite of markers is employed then eventually large numbers of research groups would be able to contribute and compare data. In this way, we could build up a national database of genetic information for koala populations, allowing ready access, analysis and comparison as new populations are added to the database. Of course, standard markers are not enough, and a standard and calibrated method of sequencing would also need to be developed to ensure comparisons were valid. A useful place to start could be the suite of microsatellites developed by Houlden et al. (1996) and Cristescu et al. (2009), used in the two studies in this thesis.

Recommendations

Based on the evidence gathered in this thesis we present the following recommendations:

1. **The Campbelltown and Heathcote koala populations should be treated as a single management unit for the purposes of management and environmental impact assessments.**
2. **The importance of the Holsworthy Army Range for maintaining a healthy Campbelltown population should be recognised when considering future uses of the Holsworthy Army Range.**
3. **Study and publish the Chlamydia status of the Campbelltown koala population, Blue Mountains and the Southern Highlands population**. Such information would have significant implications for future management of the populations.
4. **Studies should be undertaken to describe the size and distribution of the northern Blue Mountains koala population**. The evidence in this thesis suggests the Blue Mountains population could be a very large and widespread population.
5. **The City of Campbelltown Council should fund the Campbelltown koala sightings hotline.** The Campbelltown koala population is an asset to the City of Campbelltown and studies to understand and protect the Campbelltown koala population were enhanced by the sightings hotline.
6. **The City of Campbelltown Council should engage with local Veterinary hospitals to investigate options to coordinate koala treatment and potentially subsidise koala treatment.** While veterinarians are generally happy to help injured wildlife some form of subsidy would recognise the significant costs associated with koala treatment and the value of koalas to the Campbelltown community.
7. **The size and distribution of the South Gippsland population should be determined.** This information would be essential for proper management of the population.
8. **The South Gippsland population should be treated as a distinct management unit with high conservation value.**
9. **The Victorian Government should consider an increased conservation status for South Gippsland koalas. The Australian Government should also consider options for determining if the South Gippsland population would be eligible for inclusion as a threatened koala population alongside populations in NSW, ACT and Queensland.**

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