



MONASH University

**Genetic data from koala scats:
method development and investigation of
a unique population in South Gippsland**

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Bachelor of Science (Honours)

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Abstract

Data to inform the conservation of wild animal populations are needed with increasing urgency due to anthropogenic influences and their effects on climatic and habitat suitability. These changes are particularly important to an Australian habitat specialist, the koala.

Genetic factors play a role in population decline and extirpation; population genetics can provide data important to conservation. Sampling of DNA from invasive (blood or biopsy) sources for genetic analyses can be difficult, both logistically and ethically, and may limit sample sizes. Such difficulties may be overcome by use of non-invasive sources of DNA such as scats. However, due to decreased DNA quantity and quality in non-invasively sourced DNA (relative to invasive sources), method optimisation is required to ensure data quality.

This thesis describes the development and optimisation of techniques for isolating DNA from koala scats and the use of these methods to investigate the wild South Gippsland koala population, which is thought to be a remnant population, not derived from the ongoing Victorian translocation program. The translocation program re-established koala populations across Victoria following a genetic bottleneck event soon after European settlement and is likely to have resulted in the homogenisation and reduction of genetic variation in Victorian koalas.

Measures of DNA quantity and quality were assessed to determine appropriate collection, storage and DNA isolation protocols. Genetic structure and diversity was investigated using a panel of 12 microsatellites and mitochondrial DNA sequencing. PCR detection and DNA sequencing of two koala pathogens, *Chlamydia pecorum* and koala retrovirus (KoRV) in DNA isolated from scats were also tested and applied to a large set of samples from South Gippsland.

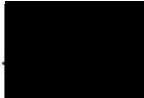
Non-invasive methods for obtaining genetic data from koala scats successfully and reliably provided information regarding genetic structure and diversity of koala populations; and pathogen prevalence. Genotypic data were obtained from koala populations in South Gippsland ($n=221$), Raymond Island, Victoria ($n=31$), Cape Otway, Victoria ($n=50$), south east New South Wales ($n=12$), north east New South Wales ($n=24$) and south east Queensland ($n=12$). The South Gippsland koala population had an additional 38 microsatellite alleles and seven mitochondrial haplotypes not present in the island derived Victorian populations, indicative of higher genetic diversity in the region. *C. pecorum* was detected in 61% of the South Gippsland population with a greater proportion of individuals carrying the bacterium in areas where koala densities were higher. In South Gippsland, KoRV was detected in 27% of individuals tested and data suggested an increased prevalence of KoRV in individuals entering shelters due to illness or trauma.

The methods presented in this thesis provide an alternative for obtaining genetic data relating to koalas and their pathogens, which will be useful to koala conservation projects. This research confirms that the koala population in South Gippsland is a remnant population; not derived from translocations of island individuals, confirming its high conservation significance. This thesis also provides important baseline data for future monitoring of genetic characteristics and pathogens in the South Gippsland koala population and measuring the effect of prospective conservation or management programs in the region.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications during enrolment

Wedrowicz F, Karsa M, Mosse J, Hogan FE (2013) Reliable genotyping of the koala (*Phascolarctos cinereus*) using DNA isolated from a single faecal pellet. *Molecular Ecology Resources* **13**, 634-641.

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Wedrowicz F, Mosse J, Wright W, Hogan F (2017) Validating the use of non-invasively sourced DNA for population genetic studies using pedigree data. *Web Ecology* **17**, 9-18.

Wedrowicz F, Wright W, Schlagloth R, Santamaria F, Cahir F (2017) Landscape, koalas and people: A historical account of koala populations and their environment in South Gippsland. *Australian Zoologist* **38**, 518-536.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes four original papers published in peer reviewed journals and three submitted publications. The core theme of the thesis is the use of non-invasively sourced DNA to study koala populations. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Faculty of Science under the supervision of Associate Professor Jennifer Mosse, Dr Fiona Hogan, Associate Professor Wendy Wright and Dr Bronwyn Isaac.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2, 3 and 5–9 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	Reliable genotyping of the koala (<i>Phascolarctos cinereus</i>) using DNA isolated from a single faecal pellet	Published	65%. Concept and collecting data and writing first draft	1) Marwar Karsa, concept and collecting data 15% 2) Jennifer Mosse, concept and input into manuscript 10% 3) Fiona Hogan, concept and input into manuscript 10%	Yes
3	Pedigree data validates the use of scat sourced DNA for population genetic studies	Published	75%. Concept, collecting data and writing first draft	1) Jennifer Mosse, concept and input into manuscript 15% 2) Fiona Hogan, input into manuscript 5% 3) Wendy Wright, input into manuscript 5%	No

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
5	Isolating DNA sourced non-invasively from koala scats: a comparison of four commercial DNA stool kits	Submitted	75%. Concept, collecting data and writing first draft	1) Fiona Hogan, input into manuscript 15% 2) Jennifer Mosse, input into manuscript 5% 3) Wendy Wright, input into manuscript 5 %	No
6	A non-invasive tool for assessing pathogen prevalence in koala (<i>Phascolarctos cinereus</i>) populations: detection of <i>Chlamydia pecorum</i> and koala retrovirus (KoRV) DNA in genetic material sourced from koala scats	Published	70%. Concept, collecting data and writing first draft	1) Tom Saxton, collecting data and input into manuscript 10% 2) Jennifer Mosse, concept and input into manuscript 10% 3) Fiona Hogan, input into manuscript 5% 4) Wendy Wright, input into manuscript 5%	Yes
7	Landscape, koalas and people: A historical account of koala populations in South Gippsland	Published	75%. Concept, research and writing first draft	1) Wendy Wright, input into manuscript 10% 2) Rolf Schlagloth, input into manuscript 5% 3) Flavia Santamaria, input into manuscript 2.5% 4) Fred Cahir, input into manuscript 2.5%	No
8	Genetic structure and diversity of the koala population in South Gippsland, Victoria: a remnant population of high conservation significance	Submitted	75%. Concept, research and writing first draft	1) Fiona Hogan, concept and input into manuscript 15% 2) Jennifer Mosse, concept and input into manuscript 5% 3) Wendy Wright, concept and input into manuscript 5%	No

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
9	Using non-invasive sampling methods to determine the prevalence and distribution of <i>Chlamydia pecorum</i> and koala retrovirus in the South Gippsland koala population	Submitted	75%. Concept, research and writing first draft	1) Tom Saxton, collecting data 5% 2) Jennifer Mosse, concept and input into manuscript 10% 3) Fiona Hogan, input into manuscript 5% 4) Wendy Wright, input into manuscript 5%	Yes

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:



Date: 28/04/2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature:



Date: 28/04/2017

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Chapter 1

Introduction





Introduction

Collecting data from wild animal populations

Obtaining ecological data from wild animal populations can be difficult when the study species is elusive, cryptic or at low population densities. When low animal densities are associated with population decline, the rapid acquisition of ecological data for the timely implementation of informed conservation strategies is imperative. The methods by which data are obtained is also an important consideration. Data collection involving animal capture may introduce an unacceptable level of risk, especially where the study population is vulnerable to extinction.

It is possible to obtain a wide range of information via microbiological, biochemical and molecular analyses of animal faeces (scats). Information obtained from scats includes the determination of parasite infections and loads (Luikart *et al.* 2008), biochemical measurement of faecal steroids for the study of reproduction and stress (Schwarzenberger 2007) and the identification of individual animals (having deposited the sample), dietary DNA (potentially including both plants and prey) or microbial DNA enabling a range of ecological questions to be answered (Beja-Pereira *et al.* 2009). Isolation of DNA from scats can be used to attain ecological information for a species using population genetics. Population genetic studies can be utilised to make inferences about the evolutionary history of a species, identify population structure, measure rates of gene flow and migration between populations, compare levels of genetic diversity, identify individuals, estimate relatedness between them and reconstruct pedigrees (Frankham *et al.* 2012).

Non-invasive genetic sampling of DNA from animal scats has several advantages over invasive sampling methods, such as blood extraction and tissue biopsy, as it allows DNA to

be obtained without having to catch and handle animals. Studies utilising well optimised methods for obtaining data from scats can minimise costs associated with sampling, maximise sample size and accelerate sample collection while at the same time providing information at a range of levels. Such methods therefore have the potential to rapidly produce large multifaceted datasets which may aid in the unravelling of factors contributing to population declines and, in turn, to inform species' conservation and management.

This project develops and validates methods for sourcing genetic material, non-invasively, for the study of koala populations using molecular methods (microsatellite genotyping, mitochondrial DNA (mtDNA) sequencing and the detection and genetic analysis of pathogens). The methods devised are then applied to the South Gippsland koala population in Victoria using conservation genetic methods and compared to reference populations both within Victoria and interstate. Results obtained from this project may help to inform the conservation and management of koala populations in Victoria.

The Koala

Koalas (*Phascolarctos cinereus*) are a native Australian marsupial, distributed throughout Australia's east (Fig. 1). In the northern Australian states of Queensland, New South Wales and the Australian Capital Territory, population declines and extirpations are of concern for the species, and koala populations in these states are classified as Vulnerable under the *Environmental Protection and Biodiversity Conservation Act 1999* (EaCRC 2012). A range of factors are believed to contribute to population declines including habitat loss, urbanisation, car strikes, dog attacks and disease (EaCRC 2011). Two pathogens infecting koala populations also play a potential role in observed declines; these are the intracellular bacterium *Chlamydia pecorum* which can lead to female infertility (Obendorf & Handasyde 1990) and the koala retrovirus (KoRV), the effects of which are not entirely clear (Tarlinton *et*

al. 2005; Kinney & Pye 2016). Genetic factors are also known to play a significant role in population declines and extinction events (Frankham 2003; Reed & Frankham 2003), although these are often overlooked by conservation management strategies and/or policy (Laikre 2010).

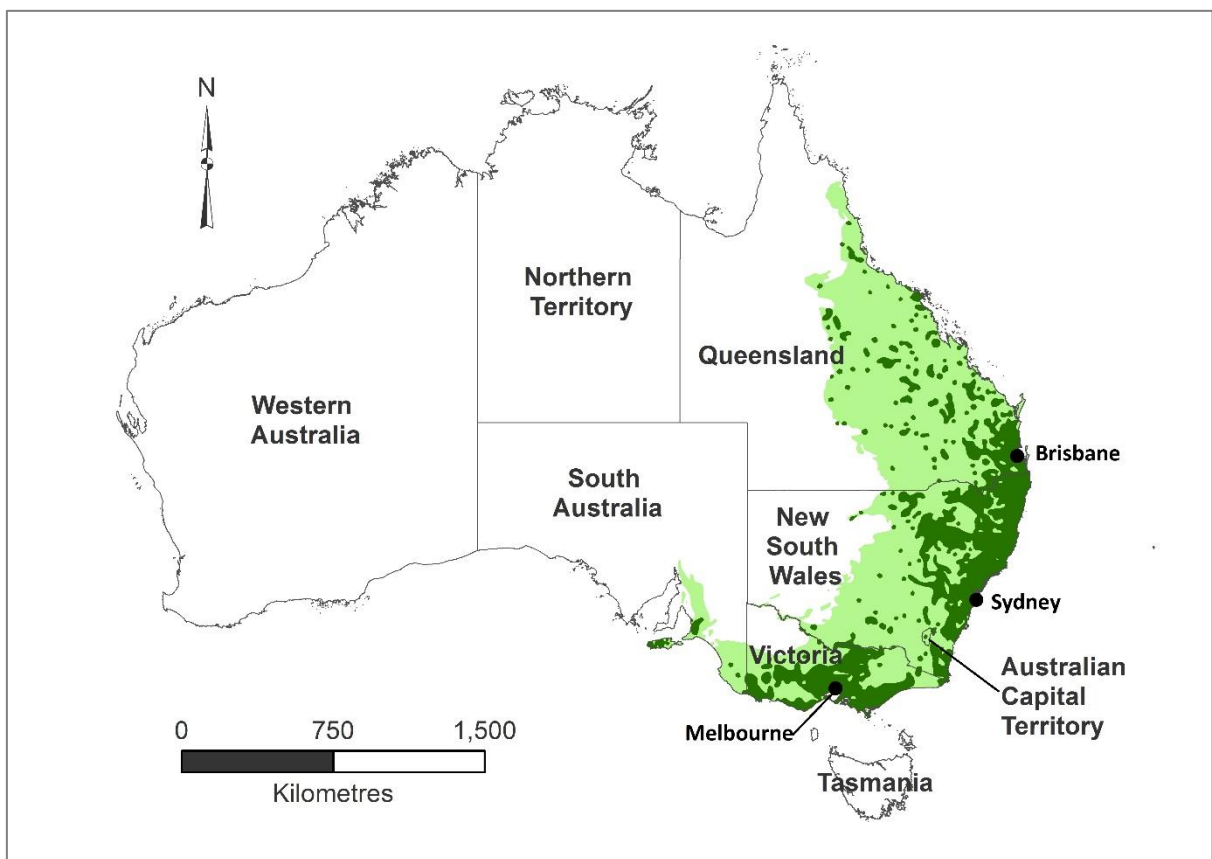


Figure 1 The distribution of the koala throughout Australia. Dark shading indicates the regions where koalas are known to occur while lighter shading indicates the extent to which koalas may occur. Distribution information adapted from Department of the Environment (2015).

In the southern Australian state of Victoria, koala conservation and management issues vary widely between populations, largely as a result of their recent history (Menkhorst 2008). Most Victorian koala populations are descendants of small numbers of koalas which were moved to Phillip and French Islands in the late 1800s (Lewis 1934, 1954). Habitat loss and hunting for the commercial fur trade occurring post-European settlement resulted in widespread extirpations of koala populations across mainland Victoria by the early 1900s (Lewis 1934, 1954). Subsequently, a government reintroduction program re-established koala populations across the state via translocations of koalas from French and Phillip Islands, which had become overpopulated by that time (Martin 1989; Menkhorst 2008). Due to the small number of founders from which they descend, re-established koala populations may have reduced levels of genetic diversity (Houlden *et al.* 1996; Houlden *et al.* 1999; Lee *et al.* 2011) and this may make these populations more susceptible to future environmental change (e.g. climate change or emerging diseases) due to a decreased ability to adapt (Frankham *et al.* 2012).

The South Gippsland koala population (Fig. 2) is thought to originate from remnant koala colonies which survived near extinction in the early 1900s, rather than from translocated island individuals (Houlden *et al.* 1999; Lee *et al.* 2011). Greater genetic diversity in the South Gippsland koala population relative to island derived populations could provide an increased capacity for this population to adapt to future environmental change. The South Gippsland koala population and its genetic diversity are therefore, potentially, of high conservation priority in the state of Victoria. Little is known, however, about the wild koala population in South Gippsland, such as its size and distribution, the amount of genetic differentiation between it and other populations, or the prevalence of pathogens such as *Chlamydia pecorum* and KoRV; such information is vital for the effective management and conservation of koala populations in a rapidly changing world.

Greater genetic diversity has been demonstrated in South Gippsland compared to French Island individuals and their descendants using nuclear DNA (microsatellites; Lee *et al.* 2011) and between South Gippsland and both French and Phillip Island koalas using mitochondrial haplotypic data (mtDNA control region; Houlden *et al.* 1999). Differences between the South Gippsland and Phillip Island koala populations have not been clearly demonstrated using nuclear DNA; with studies that compared the two populations finding no significant difference (Houlden *et al.* 1996; Fowler *et al.* 1998). The Phillip Island koala population is thought to have been established by a greater number of individuals than the French Island population (possibly 10-30 for the Phillip Island population compared to only three for the French Island population; Lewis 1954), which may have conferred a greater amount of diversity to the Phillip Island population compared to the French Island population. The few documented koala translocations to the South Gippsland area were mainly from Phillip Island (Martin 1989). Determining whether the South Gippsland koala population is a remnant Victorian population, descended from koalas which survived in the region during their near extinction in the early 1900s, or descended from Phillip Island individuals is important since uncertainty around these issues may hinder or obstruct decisions relating to legislation and/or management. Questions pertaining to the origins and structure of the South Gippsland koala population may be answered using molecular methods.

Genetic variation in wild populations

Genetic methods can reveal information regarding both the long and short term history of a species. Contemporary genetic structure and diversity of a species is the product of environmental pressures, population movements and interactions occurring across many thousands of years (Hewitt 2000). For example, range contractions occurring because of major climatic changes during glacial periods and subsequent re-expansion ,during

interglacial periods, have shaped genetic structure and diversity due to the effects of genetic drift during isolation in refugia and founding events during recolonisation (Hewitt 1999). Long term climatic changes having an effect on species would have occurred many times throughout the distant past. DNA mutations occurring across these timeframes have produced genetic variation within species that have subsequently been moulded, over time, by a range of additional factors including population size, isolation and rates of gene flow (Frankham *et al.* 2012).

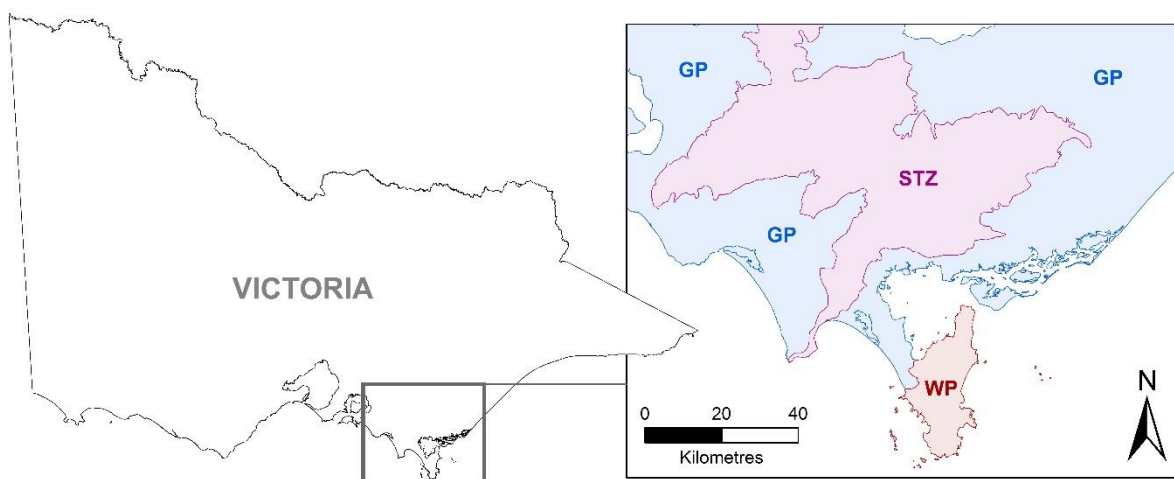


Figure 2 Map showing the location of the South Gippsland region of Victoria. The area consists of three bioregions: the Strzelecki Ranges (STZ), the Gippsland Plain (GP) and the Wilsons Promontory (WP) bioregions.

In contrast to the extended timescales required to generate diversity by mutation, environmental changes or human impacts can result in the rapid loss of diversity. The large scale loss of forest habitats in Australia after European settlement (Bradshaw 2012) is likely to have irreversibly altered the evolutionary trajectories of species reliant on those habitats

and, potentially, their capacity to adapt and survive further future challenges. Populations that might have been continuous (a single large population) or contiguous (discrete population groups connected by migration) prior to European colonisation may now exist on habitat 'islands' within the landscape, between which gene flow is greatly restricted with concomitant reductions in genetic diversity. Conservation genetic studies demonstrate positive correlations between population size, genetic diversity and fitness (Reed & Frankham 2003; Frankham *et al.* 2012). Fragmentation of a single large population into numerous small populations can therefore lead to loss of genetic diversity, resulting in higher rates of inbreeding, reduced fitness and an increased risk of extirpation for each isolated group. Increasing connectivity and gene flow between historically connected habitats are therefore key to improving future conservation outcomes for wildlife populations (Westemeier *et al.* 1998; Madsen *et al.* 2000; Hedrick ; Bouzat *et al.* 2009). Identifying the spatial distribution of population structure, genetic diversity and gene flow can therefore provide information that is essential for conservation strategies (Houde *et al.* 2015; Mijangos *et al.* 2015; Jordan *et al.* 2016). Obtaining sufficient sample sizes are, however, essential for the robust inference of population structure and genetic diversity (Hoban *et al.* 2013), though sampling methods involving animal capture can limit the sample size that can be obtained. Factors limiting sample size when using animal capture and invasive methods to source DNA can be reduced using non-invasive genetic sampling to source DNA samples.

Non-invasive genetic sampling

Koalas can be difficult to sample due to their favoured position in the tops of eucalypt trees that can be over 30 metres tall; non-invasive methods of sampling are therefore ideal for this species. Non-invasive genetic sampling can offer a range of potential advantages over traditional methods of sampling DNA (e.g. tissue biopsies or the collection of blood) which

are summarised in Table 1. A major disadvantage of DNA isolated from non-invasively collected samples is a general reduction in the quantity and quality of DNA obtained due to lower numbers of cells available for DNA isolation and DNA degradation (Taberlet *et al.* 1996; Pompanon *et al.* 2005). The polymerase chain reaction (PCR) is a major step used in most molecular analyses to amplify target DNA. The main issues arising from reduced DNA quantity and quality are failed PCR amplification and genotyping errors, which can result in incorrect inference of gender and inaccurate DNA profiles used to identify individuals. Issues such as these have the potential to result in inaccurate data and possibly the implementation of inappropriate management plans based on erroneous results. Laboratory work using non-invasively sourced DNA will therefore normally require optimised lab protocols and replicate analyses to ensure data reliability.

Amplification failure

PCR is an incredibly sensitive technique that is theoretically capable of amplifying a single target molecule, but this is not always the case and low numbers of target DNA may fail to amplify due to chance (Taberlet *et al.* 1996). The quantity and quality of the DNA template used for analysis is a major determinant of PCR success. PCR failure is typically associated with DNA degradation and/or the presence of PCR inhibitors. Most DNA degradation occurs via the action of nucleases which may originate from the dead cell itself or from environmental microbes (Alaeddini *et al.* 2010). Oxidative reactions may also cleave DNA, or introduce base modifications; both will block DNA amplification (Deagle *et al.* 2006; Alaeddini *et al.* 2010). PCR inhibitors impede amplification by binding or degrading target DNA, primer DNA and/or the polymerase (Wilson 1997). Plant molecules such as polysaccharides and tannins (Wilson 1997) originating from the koalas eucalypt diet along with metabolic wastes such as bile salts and bilirubin (Widjojoatmodjo *et al.* 1992; Wilson

1997) may contaminate DNA isolates and have an inhibitory effect on amplification. The effect of PCR inhibitors can often, however, be alleviated by use of amplification facilitators such as bovine serum albumin (BSA) in PCR mixes (Abu Al-Soud & Rådström 2000).

Genotyping errors

Genotyping errors in final datasets can lead to erroneous findings as a result of homozygote excesses, potentially leading to an overestimation of inbreeding or population size and inaccuracies in parentage analysis and individual identification (Bonin *et al.* 2004); such errors are more likely in DNA obtained using non-invasive sampling methods (Golenberg *et al.* 1996; Taberlet *et al.* 1999). Genotyping errors are noted when two or more genotypes appear to have originated independently from the same sample; the major error types being allelic dropout and false alleles (Fig. 3; Taberlet *et al.* 1996; Pompanon *et al.* 2005). Allelic dropout occurs when only one of the two alleles in a heterozygote is amplified, while false alleles are amplified PCR artefacts that may be mistaken for a true allele (Pompanon *et al.* 2005). Allelic dropout can arise when DNA fragmentation has occurred, or when DNA quantity is low, as a result of stochastic sampling error; the chance that only one of the two allele templates will be added to the reaction mix (Taberlet *et al.* 1996; Morin *et al.* 2001). Differential denaturation of alleles can also result in allelic dropout via the preferential amplification of shorter alleles (Walsh *et al.* 1992). False alleles may appear due to the presence of contaminating DNA, which have a greater chance of representation in non-invasively collected samples that have similarly low concentrations of target DNA (Morin *et al.* 2001). Early polymerase slippage events in low quantity samples can generate false alleles at similar levels to that of the true allele (Taberlet *et al.* 1996), while highly fragmented DNA has the potential to form chimeric alleles, in which staggered annealing of fragments derived from two different alleles with complementary sequences are amplified (Golenberg *et al.*

1996; Beja-Pereira *et al.* 2009). Rates of allelic dropout and false alleles can be detected and assessed by carrying out replicate genotyping, by analysing known pedigrees, or by comparing genotypes with those obtained from high quality sources (Bonin *et al.* 2004; Pompanon *et al.* 2005).

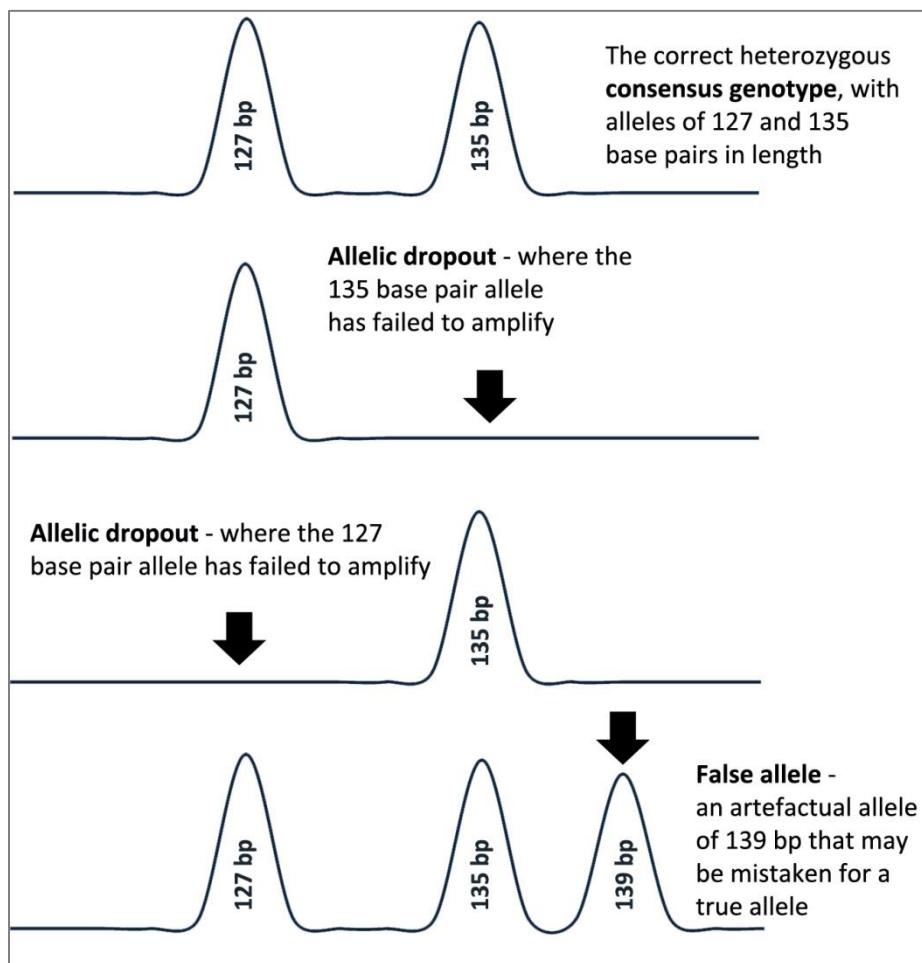


Figure 3 The main errors that can occur during genotyping. Adapted from Taberlet *et al.* (1996) and Pompanon *et al.* (2005).

Although there are a number of potential challenges to obtaining DNA non-invasively, the benefits of being able to study wild animal populations non-invasively as well as the associated savings in both time and money, makes any extra work involved in method development worthwhile. Overcoming challenges might involve devising sampling and storage strategies that maximise the quality of starting material (Piggott & Taylor 2003); optimising DNA isolation methods to maximise DNA recovery and minimise further degradation; screening samples for DNA quantity and quality before conducting further analysis (Hogan *et al.* 2008) and using replicate analyses (Taberlet *et al.* 1996; Valière *et al.* 2007), measures of data quality (Miquel *et al.* 2006) and error checking strategies (Paetkau 2003) to maximise reliability in final datasets. Once established, protocols for the isolation and analysis of non-invasively sourced DNA need not be limited to particular uses, but can continue to be refined and extended to new methods of analysis as they become viable.

Table 1 Potential benefits of non-invasive sampling (e.g. scats) over invasive sampling methods (e.g. blood or biopsies)

Invasive DNA sampling (e.g. blood or biopsies)	Non-invasive DNA sampling (e.g. scats)
Animal ethics – capture is stressful for animals. There is a potential for health risks associated with capture and invasive procedures	Animal stress is minimised and potential impacts on animal health are eliminated
Need for specialist expertise/equipment and/or veterinarian assistance	Samples can be collected by anyone that can confidently identify the scats of interest. Community involvement (e.g. citizen science) can increase sample size and geographic spread of samples obtained. Training in scat identification may be required
Time consuming and costly – due to need to catch individual koalas which requires tree climbers and veterinary expertise	Time and monetary costs of fieldwork can be greatly decreased using non-invasive sampling as no specialist equipment or expertise is needed
Accessibility can be limited by remote or rugged terrain	Eliminating the need for animal capture also reduces accessibility issues as difficult terrain can be more easily traversed on foot without the range of equipment that may be required for animal capture
Sample size may be limited by the number of individuals that can be successfully caught and sampled during fieldwork. Sample sizes are also limited by ethics applications defining the number of individuals that can be caught	Eliminating the need to capture animals increases the time that can be spent collecting samples from the forest floor
Locating animals for capture can be difficult in low density populations	The ability to sample scats without locating the animal increases the chance that samples will be obtained. The chance of retrieving scats from low density populations can be increased using detector dogs
Behaviour could be impacted potentially influencing study results	Potential effects of animal interference are eliminated
Opportunistic sampling (e.g. roadkill) may lead to sample biases. For example, the more mobile gender or animals that are unwell may be overrepresented in road killed individuals	Sampling biases may be reduced or eliminated

Aims and thesis outline

The overarching aim of this project is to develop robust and reliable non-invasive methods for studying koala populations and to apply these to the wild koala population in South Gippsland, Victoria. The outcomes from this study will provide 1) methodologies for studying koala populations non-invasively and 2) information about the wild South Gippsland koala population, such as its genetic characteristics and prevalence of infections, with the potential to impact koala health, in relation to other koala populations in Victoria, New South Wales and Queensland.

This thesis is presented in two sections. The first part of this thesis is dedicated to the development, optimisation and validation of methods for obtaining genetic data from koala scats; that will be applied in the second part of the thesis, which uses data gained from koala scats to obtain information about the South Gippsland koala population. Part one (chapters two to six) aims to devise and validate methods for obtaining DNA of sufficient quantity and quality for reliable microsatellite genotyping, gender identification, DNA sequencing and the detection of pathogens important to koala health; *Chlamydia pecorum* and koala retrovirus (KoRV). The major objectives of part two (chapters seven to ten) of this thesis are to characterise the South Gippsland koala population using genetic data sourced from scats. Population genetic techniques will then utilise the genetic data to compare genetic structure and diversity between populations. DNA sampled from wild koala populations will be also be used to determine the incidence of *C. pecorum* and KoRV.

Part 1 / Method development, optimisation and validation

Determining the rates of error associated with DNA isolated non-invasively from a particular species and source is an essential first step for any study utilising non-invasive genetic sampling. **Chapter two** (Wedrowicz *et al.* 2013), therefore, describes the development of a

method for isolating DNA from koala scats. Replicate genotyping was used to estimate error rates, which were found to be relatively low. Simulated data using the determined error rates, provided an indication of the number of replicate genotypes required to obtain a reliable DNA profile using twelve microsatellite markers (Chapter two; Wedrowicz *et al.* 2013). Some errors, such as null alleles, may however, remain undetected using replicate genotyping.

Consequently, **chapter three** (Wedrowicz *et al.* 2017a) validates the number of replicate genotypes (inferred by simulations) and scoring methods devised in chapter two by analysing empirical data from a multigenerational pedigree. The power of the marker set to unequivocally discriminate individuals by assessing the frequency at which closely related individuals would have identical or near matching genotypes was also assessed.

Relatedness estimators are a commonly used tool in population genetic studies for which accuracy can vary widely depending on the study system and chosen estimator. Choosing relatedness estimators based on evaluations of performance is therefore important, but rarely undertaken (Taylor 2015). The availability of pedigree data (used for chapter three) also provided an opportunity to assess the performance of different estimators to infer relatedness, which is reported in **chapter four**.

During the second year of this study (2014) the commercial DNA isolation kit used in our methods became unavailable. **Chapter five** (Wedrowicz *et al.* in review 1) therefore compares the performance of a number of commercial DNA extraction kits to identify alternatives suitable for use with our methods; it was found that the performance of DNA isolates can vary substantially depending on the commercial DNA kit used.

DNA isolated from koala scats is likely to contain dietary (eucalypt) and microbial DNA as well as koala DNA. In **chapter six** (Wedrowicz *et al.* 2016), the applicability and range of

information obtained from DNA isolated from koala scats is extended to include the detection of infections with *C. pecorum* and KoRV.

Part 2 / Investigating the South Gippsland koala population using molecular methods

Genetic structure and diversity of populations are the result of both distant and recent past events. Rapid landscape change and population declines occurring after European colonisation in Victoria are likely to have left their mark on the genetic structure of many wild animal populations. The history of koala populations in South Gippsland along with anthropogenic and landscape factors affecting them since European settlement is explored in **chapter seven** (Wedrowicz *et al.* 2017b), in order to gain an appreciation of factors likely to have shaped koala population structure in the recent past.

In **chapter eight** (Wedrowicz *et al.* in review 2), questions regarding population structure and genetic diversity in the South Gippsland koala population are investigated at a broad (south east Queensland to Victoria), state (Victoria) and fine (South Gippsland) scale using both microsatellite genotype data and mtDNA sequence data.

In **chapter nine** (Wedrowicz *et al.* in review 3) the prevalence and geographic distribution of *C. pecorum* and KoRV detected in DNA isolated from koala scats is determined, revealing contrasting spatial patterns of infected individuals.

The final chapter in this thesis, **chapter ten**, presents a discussion of the outcomes of this project as a whole highlighting the value of non-invasive sampling schemes and discussing potential implications of this research for koala conservation.

Thesis chapters | part one

Chapter 2 (Wedrowicz *et al.* 2013)

Wedrowicz F, Karsa M, Mosse J, Hogan FE (2013) Reliable genotyping of the koala (*Phascolarctos cinereus*) using DNA isolated from a single faecal pellet. *Molecular Ecology Resources* **13**, 634-641.

Chapter 3 (Wedrowicz *et al.* 2017)

Wedrowicz F, Mosse J, Wright W, Hogan FE (2017) Validating the use of non-invasively sourced DNA for population genetic studies using pedigree data. *Web Ecology* **17**, 9-18.

Chapter 4 (Wedrowicz *et al.* in preparation 1)

Wedrowicz F, Mosse J, Wright W, Hogan FE The performance of relatedness estimators for wildlife studies: an evaluation using empirical and simulated data.

Chapter 5 (Wedrowicz *et al.* in review 1)

Wedrowicz F, Mosse J, Wright W, Hogan FE Isolating DNA sourced non-invasively from koala scats: a comparison of four commercial DNA stool kits. *Conservation Genetics Resources*

Chapter 6 (Wedrowicz *et al.* 2016)

Wedrowicz F, Saxton T, Mosse J, Wright W, Hogan FE (2016) A non-invasive tool for assessing pathogen prevalence in koala (*Phascolarctos cinereus*) populations: detection of *Chlamydia pecorum* and koala retrovirus (KoRV) DNA in genetic material sourced from scats. *Conservation Genetics Resources* **8**, 511-521.

Thesis chapters | part two

Chapter 7 (Wedrowicz *et al.* 2017)

Wedrowicz F, Wright W, Schlagloth R, Santamaria F, Cahir F (2017) Landscape, koalas and people: A historical account of koala populations and their environment in South Gippsland. *Australian Zoologist* 38, 518-536.

Chapter 8 (Wedrowicz *et al.* in review 2)

Wedrowicz F, Mosse J, Wright W, Hogan FE Genetic structure and diversity of the koala population in South Gippsland, Victoria: a remnant population of high conservation significance. *Conservation Genetics*

Chapter 9 (Wedrowicz *et al.* in review 3)

Wedrowicz F, Mosse J, Wright W, Hogan FE Using non-invasive sampling methods to determine the prevalence and distribution of *Chlamydia pecorum* and koala retrovirus in the South Gippsland koala population. *Wildlife Research*

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Part 1

Method development, optimisation and validation





Chapter 2 | foreword

DNA for genetic studies of wild animals can be sourced by invasive sampling, where an animal is caught for samples to be collected (e.g. tissue biopsy or blood) or by non-invasive sampling, where biological material such as feathers, hair or faecal pellets discarded by the host are collected. Non-invasive sampling offers a great alternative to invasive sampling, as animals don't need to be caught or even seen to be sampled. However, non-invasively sourced DNA, may be somewhat degraded and as such, DNA quantity and quality is often reduced.

Pilot studies are therefore an important first step when using non-invasively sourced DNA for genetic studies. Pilot studies aim to optimise procedures such as sample collection and storage and DNA isolation protocols to minimise rates of amplification failure and genotyping error. Estimation of error rates is needed to determine the number of times each genotype should be replicated in order to provide a high level of confidence in consensus genotypes.

The main objectives of chapter two were therefore to determine appropriate methods for scat collection and storage, to estimate the rates of error associated with DNA isolated from koala scats and to ascertain the number of times each genotype should be replicated to ensure highly reliable data.

The fundamental laboratory work for chapter two was carried out during two undergraduate honours projects undertaken by Marwar Karsa¹ and myself². This work was written up for publication after completion of both projects; and as one of the first steps in my PhD candidature. Chapter two is included as the initial chapter in this thesis, as it forms the basis for those that follow.

Three main findings in chapter two directed our methods for collection and genotyping in the remainder of the study. The first was that collection of koala scats in paper bags resulted in an increase in amplification failure and genotyping errors. This informed subsequent

¹ Karsa MM (2007) *Genetic analysis of koala populations using DNA extracted from faecal material* Honours thesis, Monash University, Churchill.

² Wedrowicz F (2012) *Non-invasive DNA sampling from scats for genetic investigation of koala (Phascolarctos cinereus) populations* Honours thesis, Monash University, Churchill.

collection protocols, whereby scats were collected and stored on toothpicks in open ended (well ventilated) containers. The second was that, when using the best identified collection and storage methods, rates of amplification failure and genotyping error were relatively low. Thirdly, chapter two determined the number of replicate microsatellite genotypes (based on the total genomic DNA concentration of the sample) needed in order to be 99.9% confident that resultant consensus genotypes would be without error.

Chapter 2

Reliable genotyping of the koala (*Phascolarctos cinereus*) using DNA isolated from a single faecal pellet



Wedrowicz F, Karsa M, Mosse J, Hogan FE (2013) Reliable genotyping of the koala (*Phascolarctos cinereus*) using DNA isolated from a single faecal pellet. *Molecular Ecology Resources* **13**, 634-641.



Reliable genotyping of the koala (*Phascolarctos cinereus*) using DNA isolated from a single faecal pellet

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Abstract

The koala, an Australian icon, has been added to the threatened species list. Rationale for the listing includes proposed declines in population size, threats to populations (e.g. disease) and loss and fragmentation of habitat. There is now an urgent need to obtain accurate data to assess the status of koala populations in Australia, to ensure the long-term viability of this species. Advances in genetic techniques have enabled DNA analysis to study and inform the management of wild populations; however, sampling of individual koalas is difficult in tall, often remote, eucalypt forest. The collection of faecal pellets (scats) from the forest floor presents an opportunistic sampling strategy, where DNA can be collected without capturing or even sighting an individual. Obtaining DNA via noninvasive sampling can be used to rapidly sample a large proportion of a population; however, DNA from noninvasively collected samples is often degraded. Factors influencing DNA quality and quantity include environmental exposure, diet and methods of sample collection, storage and DNA isolation. Reduced DNA quality and quantity can introduce genotyping errors and provide inaccurate DNA profiles, reducing confidence in the ability of such data to inform management/conservation strategies. Here, we present a protocol that produces a reliable individual koala genotype from a single faecal pellet and highlight the importance of optimizing DNA isolation and analysis for the species of interest. This method could readily be adapted for genetic studies of mammals other than koalas, particularly those whose diet contains high proportions of volatile materials that are likely to induce DNA damage.

Keywords: faecal pellet, genotyping error, mammal, microsatellite, mitochondria, sex

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Introduction

The koala (*Phascolarctos cinereus*), an endemic Australian icon, is widely distributed along eastern Australia from northern Queensland to South Australia, where it inhabits *Eucalyptus* forests and bushland (Martin & Handasyde 1999). The combined populations of Queensland, New South Wales and the Australian Capital Territory have recently been listed as Vulnerable under the *Environmental Protection and Biodiversity Conservation Act 1999* (effective of 12 May 2012). Threats to populations include disease (*Chlamydia* and koala retrovirus), dog attacks, cars, bushfires, drought and the loss and fragmentation of suitable koala habitat (EaCRC 2011).

There is now an urgent need for reliable data to assess genetic diversity, predict population trends and forecast the long-term viability of all Australian koala populations. Collecting census data or genetic samples can be difficult, especially when species abundance is low and

terrain conditions are challenging. To date, genetic studies of koala populations have relied primarily on collection of blood or tissue samples (Worthington-Wilmer *et al.* 1993; Houlden *et al.* 1996b; Lee *et al.* 2011). Obtaining such samples by animal capture is labour intensive, costly, stressful for the individual animals and raises questions of research ethics for both the scientific community and general public (Swart 2004). Noninvasive genetic sampling, where DNA is recovered from discarded sources such as shed hair, faecal pellets and feathers (Waits & Paetkau 2005), is an attractive alternative to tissue sampling. These techniques provide an opportunity to obtain genetic material from free-ranging animals in their natural environment, without having to catch, handle or even observe them (Taberlet *et al.* 1999). Noninvasive sampling is especially valuable when studying species that are rare, elusive or difficult to find and capture (Piggott & Taylor 2003b), such as koalas, who inhabit trees that may be over 30 m tall.

Opportunistic collection of faeces has been shown to be a reliable method of obtaining DNA from animals in the wild (Constable *et al.* 1995; Gerloff *et al.* 1995; Luikart

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et al. 2008). By recovering DNA from the epithelial cells that have been exfoliated onto the surface of a faecal pellet, wild animals can be sampled unobtrusively and without the sampling biases inherent in other opportunistic approaches. Faecal samples have been used as a source of DNA for assessing the population structure, breeding behaviour, habitat use and home range of a variety of mammals including the brown bear (*Ursus arctos*) (Kohn *et al.* 1995), dugong (*Dugong dugon*) (Tikel *et al.* 1996), seals (*Halichoerus grypus* and *Phoca vitulina*) (Reed *et al.* 1997), baboons (*Papio anubis*) (Constable *et al.* 1995) and wombats (*Vombatus ursinus*) (Banks *et al.* 2002). DNA isolated from faecal samples, however, may be associated with increased rates of amplification failure and genotyping error, in particular allelic dropout and false alleles (Bonin *et al.* 2004; Pompanon *et al.* 2005). Genotyping reliability may also be affected by scat storage (Frantzen *et al.* 1998; Piggott & Taylor 2003a; Soto-Calderon *et al.* 2009), sampling (Lampa *et al.* 2008; Stenglein *et al.* 2010) and isolation methods (Wehausen *et al.* 2004).

Koala scats are easily distinguishable from those of other species by their characteristic shape, colour and strong eucalypt odour. Scats have been used to estimate koala distribution, abundance and tree use (Jurskis & Potter 1997; Sullivan *et al.* 2002, 2004), but their use in population genetic studies has not been reported to date. Isolation and amplification of DNA from koala scats may be hindered by the presence of volatile organic compounds and phenolics deriving from the koala's sole diet of *Eucalyptus* leaves. Eucalypt molecules such as α -pinene, 1,8-cineole and terpinene-4-ol are excreted in koala faeces (Eberhard *et al.* 1975) and are known to damage cell membranes (Carson *et al.* 2006), while phenolics could accelerate DNA degradation (Khan & Hadi 1998) and/or inhibit the polymerase chain reaction (PCR) process (Kreader 1996), thereby reducing amplification success.

Here, we present a method that can be used to unequivocally identify an individual koala from a single faecal pellet. This study addressed methods for sampling and storage of koala scats to maximize DNA yield and genotyping reliability, and the application of genetic markers (microsatellite and gender) to provide a DNA profile that will confidently identify an individual koala.

Materials and methods

Scat collection and storage

Fresh (<24 h) koala scats were collected from captive koalas at the Phillip Island Nature Park, Victoria, Australia ($n = 40$). To determine the effect of sample age and weather, scats ($n = 16$) were placed outside in a position where some were exposed to all weather conditions,

including rain and wind, while others remained in a sheltered position that provided sun exposure, but protection from rain. DNA was isolated from two scats in each group at 1, 2, 3 and 4 weeks.

To consider the effect of storage conditions, fresh scats were placed into individual paper bags immediately upon collection ($n = 12$) or sprayed with 70% ethanol, allowed to air-dry and then placed into individual paper bags ($n = 12$). Bagged samples were stored, undisturbed, at room temperature, and DNA was isolated from three scats in each group at 1, 2, 4 and 6 weeks after collection.

DNA yield and genotyping optimization

Koala scats ($n = 270$) were collected from wild koalas in temporary care at the Southern Ash Wildlife Shelter, Rawson, Victoria, and captive koalas at Maru Koala and Animal Park, Grantville, Victoria. Scat collection was undertaken within 24 h after pens had been cleaned to ensure consistency in the age of the scats sampled. Scats were collected on wooden toothpicks and stored uncovered for between 6 and 54 h, at ambient temperature in the laboratory until DNA isolation. A subset of fresh scats ($n = 36$) was stored for 1, 2, 4, 6, 8 and 10 weeks (six scats for each time period) before DNA isolation. Each scat was weighed and measured (length and width) prior to DNA isolation.

Isolation of koala DNA

Intestinal epithelial cells on the surface of the scat were isolated by placing individual koala scats in 7 mL vials with 2 mL of phosphate-buffered saline (PBS) which were rolled on a gyratory mixer for 8 min. All of the wash was transferred to a 2 mL microcentrifuge tube avoiding the transfer of visible debris. Cells within the surface wash were pelleted by centrifuging at 2500 g for 5 min. The majority of the supernatant was discarded leaving behind approximately 175 μ L of the wash and the pellet. The remaining wash (~175 μ L) was thoroughly vortexed (~30–60 s) to resuspend the pellet. DNA was then isolated from collected cells using the QIAamp[®] DNA stool mini kit (Qiagen) following the manufacturer's protocol for the isolation of DNA from stool for human DNA analysis, with modification of the cell lysis steps: 1400 μ L of Buffer ASL was added to the wash followed by incubation for 1 h at 35 °C with periodic vortexing (~15 s) each 15–20 min. DNA was eluted in 100 μ L ($2 \times 50 \mu$ L) using 5-min incubation periods. The amount of total DNA (koala and foreign) obtained was determined fluorometrically; isolates were stored at –20 °C.

To confirm the presence of koala DNA, a 890 bp region of the mitochondrial control region was amplified using primers KmtL2 and KmtH2 (Fowler *et al.* 2000).

Amplification reactions consisted of 5 μ L of GoTaq[®] Green Master Mix (Promega), 1 μ g of BSA, 0.25 μ M each of KmtL2 and KmtH2 and 1 μ L of DNA template in a total volume of 10 μ L. PCR was carried out using an initial denaturation of 3 min at 95 °C, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min followed by a final 5 min extension at 72 °C.

To assess DNA quality, isolates from the scat collection and storage trial were amplified using nine koala microsatellite markers: K10.1, Pcv2, Pcv6.3, Pcv24.2, Pcv30, Pcv31 (Cristescu *et al.* 2009), Phc2, Phc4 and Phc13 (Houlden *et al.* 1996a), where markers were amplified six times per isolate. DNA isolates for the DNA yield and genotyping optimization trial were amplified using 12 koala microsatellite markers, which included all of the markers listed above plus the addition of K2.1, Pcv6.1 and Pcv25.2 (Cristescu *et al.* 2009), where each marker was amplified eight times for each isolate. Scats stored for 1–10 weeks were genotyped for the 12 koala microsatellite loci using the number of replicates predicted by GEMINI, according to isolate concentration. Resulting DNA profiles were compared to those obtained from fresh scats. Genotyping was carried out on the Applied Biosystems 3730 DNA analyser and GENEMAPPER 3.7 software (Applied Biosystems) by Australian Genome Research Facility, Melbourne, Australia.

Amplification success and genotyping errors

The error rates estimation calculator in GIMLET v 1.3.3 (Valière 2002) was used to calculate the rates of amplification and genotyping error using allelic frequencies determined in GENALEX 6.5 (Peakall & Smouse 2012). GIMLET defines amplification success as the number of successfully amplifying loci divided by the total number of loci for which amplification was attempted. Consensus genotypes were generated using the threshold rule, where an allele must appear at least twice to be accepted. Using the error rates calculated, the PCR repetition batch module of GEMINI v 1.3.0 (Valière *et al.* 2002) was employed to estimate the minimum number of replicates required to obtain reliable genotypes. Replicate simulations were run ($n = 250$), using hypothetical populations consisting of 20 individuals, taking 50 samples (with replication) on one sampling occasion. Regression analysis and statistical comparisons between amplification and error rates obtained were carried out using *t*-tests, ANOVA or the general linear model using Minitab[®] 15 Statistical Software (Minitab 2007).

Sexing markers

Marsupial sexing markers were tested for amplification success in the koala using Y-linked primers (IMY1 and

IMY2) designed to amplify a 159-bp region of sex determining region of the Y chromosome (SRY) sequence in the northern brown bandicoot (*Isodon macrourus*) (Watson *et al.* 1998) and X-linked primers (GpdEx12 and GpdEx13R) developed from wallaroo (*Macropus robustus*) sequence to amplify 175 bp of DNA within the G6PD (glucose-6-phosphate dehydrogenase) gene (Loebel *et al.* 1995; Loebel & Johnston 1997). Y-linked DNA was amplified in a single reaction along with the X-linked G6PD region as an internal control. Reactions comprised 5 μ L of GoTaq[®] Green Master Mix, 1 μ g of BSA, 0.2 μ M each of IMY1 and IMY2, 0.3 μ M each of GpdEx12 and GpdEx13R and 1 μ L of DNA template, adjusted to 10 μ L with water. Thermal cycling began with an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s and finished with one final extension cycle at 72 °C for 4 min.

Results

Scat collection and storage

Exposure to weather—Where scats were exposed to all weather conditions, there was a significant decrease in both DNA concentration (from 0.50 ng/ μ L at week 1 to 0.05 ng/ μ L at week 4) and PCR amplification rates (from 62% at week 1 to 1.0% at week 4) over time of exposure. In contrast, PCR amplification rates remained steady ($90 \pm 4.6\%$) in the sheltered treatment group and mean genotyping reliability across sheltered samples was consistently high (allelic dropout: $1.0 \pm 1.7\%$; false alleles: $2.1 \pm 2.8\%$). GEMINI simulations indicated that three replicates would be required for reliable genotyping using scats, up to 4 weeks old, stored outside in a sheltered position (Table 1). Koala scat collection should therefore be conducted during a period of dry weather.

Storage methods—Spraying koala scats with 70% ethanol before storage in paper bags did not produce a difference in amplification success or genotyping error rates compared to storage in paper bags alone. Storage in paper bags, with or without ethanol treatment, did, however, provide less reliable genotypes compared to isolates obtained from scats left outside, protected from rain (sheltered group). Amplification success was 24% greater ($P < 0.0005$) and allelic dropout 17% lower ($P = 0.001$) for samples stored outside (sheltered group) compared to those stored in paper bags (Table 1).

Differences in DNA concentration were not significant between scats stored in paper bags and those stored in an open sheltered position. This indicates that the reduced genotyping reliability observed for samples obtained from scats stored in paper bags was not associated with the loss of cells (and thus available DNA) onto

Table 1 The effect of sample storage conditions on amplification success and genotyping error (average across loci)

Treatment*	Mean DNA concentration \pm 95% CI (ng/ μ L)	Amplification success (%)	Allelic dropout (%)	False alleles (%)	Number of replicates† (% correct id)
Paper bag	1.3 \pm 1.3	66	18	0.20	5 (99.7)
Paper bag (ethanol)	0.88 \pm 0.49	63	18	0.00	5 (99.7)
Outside	0.28 \pm 0.17	33	33	0.30	6 (99.4)
Outside (sheltered)	1.6 \pm 0.63	90	0.80	0.40	3 (99.9)

CI, confidence interval.

*Scats from each treatment ($n = 8$) stored for 4 weeks or less.

†The number of replicates required to be confident of obtaining the correct genotype (percentage of correctly assigned genotypes) calculated using GEMINI (Valière 2002).

the surface of the paper bag. It may be that volatile components originating from the eucalypt leaves consumed by koalas could reach increased concentrations within the confines of the closed paper bag, potentially exerting a negative effect on surface cells and DNA and/or increasing carryover of inhibitory molecules. Collecting koala scats in bags (paper or otherwise) is therefore not ideal and should be avoided.

DNA yield and genotyping optimization

DNA quantity—DNA concentration of isolates obtained from single scats ranged from 0.08 to 120 ng/ μ L ($\bar{x} = 6.1 \pm 1.5$ ng/ μ L). Scats that were <30 h old provided higher DNA yields ($\bar{x} = 11 \pm 1.6$ ng/ μ L) than older scats ($\bar{x} = 2.2 \pm 0.31$ ng/ μ L) ($P < 0.0005$), see Fig. 1. The presence of koala DNA was confirmed by successful amplification of the mitochondrial control region.

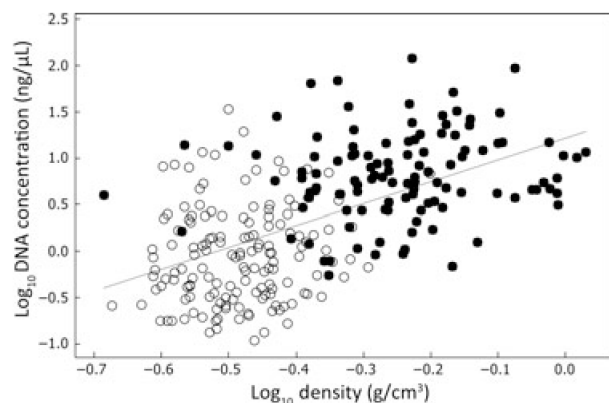


Fig. 1 Correlation between scat density and DNA concentration grouped according to scat age at the time of isolation; scats <30 h old (●, $n = 114$) and scats more than 30 h old (○, $n = 156$). The regression equation is $\text{Log}_{10}\text{DNA} = 1.22 + 2.36 \text{Log}_{10} \text{Density}$ ($R^2 = 31.9\%$, $P < 0.0005$).

A positive correlation was found to exist between scat density and DNA concentration (Fig. 1), with higher DNA yields being obtained from scats with higher densities, most likely due to the evaporation of moisture from the scat. Scats dry out rapidly; scat density is reduced by $45 \pm 5.5\%$ after 30 h, with no evidence of further reductions in density past this point. Scat density could be used to determine the freshest scats, which will likely yield higher DNA concentrations. Because scats from an individual koala differ little in size, weight alone could be used to indicate the most recent scats from one animal.

Amplification success and genotyping error—Rates of amplification success and genotyping error were calculated using 21 samples whose DNA concentrations increased incrementally from 0.25 to 20 ng/ μ L. Amplification success increased from 87% for isolates containing 0.25–1.0 ng total DNA per reaction, to 95% for samples with 1.0–5.0 ng DNA, to 100% in samples providing more than 5 ng total DNA per reaction. Average chromatograph peak height also significantly ($P < 0.0005$) increased from 4315 ± 305 where samples provided 0.25–1.0 ng total DNA per reaction, to 6050 ± 321 for samples with 1.0–5.0 ng DNA, to 9375 ± 510 in samples having more than 5 ng total DNA per reaction, indicating that koala specific PCR product increases with increasing total (koala and foreign) DNA levels. Allelic dropout and false alleles both decreased from 7.5% to 2.7%, respectively, in samples with 0.25–1.0 ng total DNA to 1.8% and 0.3%, respectively, in samples supplying more than 1.0 ng total DNA per reaction (Table 2). GEMINI simulations indicated that four replicates would be required for amplifications with 0.25–1.0 ng total DNA/reaction, while three replicates would be sufficient for samples containing more than 1.0 ng total DNA.

Scat storage and genotyping reliability—DNA samples isolated from scats stored for up to 10 weeks were found to give complete genotypes, identical to genotypes

Table 2 Error rates of the microsatellite loci used for genotyping the koala grouped according to the amount of total DNA per reaction

Locus	Largest allele (bp)	0.25–1.0 ng DNA/reaction			1.0–20 ng DNA/reaction		
		+PCR (%)	ADO (%)	FA (%)	+PCR (%)	ADO (%)	FA (%)
Phc4	123	100	0	0	99	0	0
Phc13	131	94	28	0	99	2.5	0
K10.1	142	94	6.8	0	95	0	0
Pcv2	144	42	10	0	88	7.5	0
Pcv25.2	178	90	6.7	0	98	0	0
K2.1	180	92	0	0	98	0	0
Phc2	197	75	0	0	91	0	3.1
Pcv30	203	92	0	0	99	1.3	0
Pcv24.2	218	96	0	2.9	100	10	0
Pcv6.1	233	96	14	12	98	0	0
Pcv31	235	92	14	0	99	0	0
Pcv6.3	306	85	9.4	17	96	0	0
Mean	—	87	7.5	2.7	97	1.8	0.3

+PCR, successful PCR amplification rate; ADO, allelic dropout rate; FA, false allele rate; Mean, average across loci.

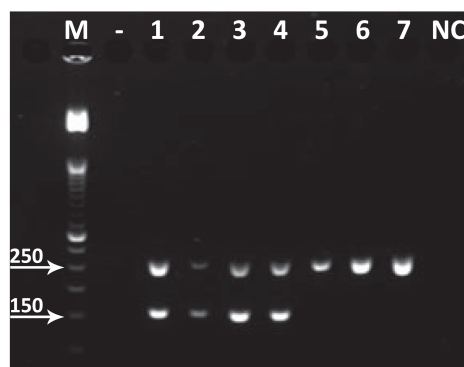


Fig. 2 Amplification of Y- and X-linked DNA markers in a single reaction to determine gender: Lane M, Invitrogen TrackIt™ 50 bp DNA Ladder, Lanes 1–3: Male koala DNA isolated from scats, Lane 4: Male koala DNA isolated from blood, Lanes 5–7: Female koala DNA isolated from scats. The Y- and X-linked markers produced amplicons of approximately 150 and 250 bp in length, respectively. NC is negative control.

obtained from fresh scats (<30 h old at DNA isolation). Where total DNA concentration of isolates was between 0.25 and 5.0 ng/ μ L, 5.5% of amplifications failed for scats <54 h old, while 28% of amplifications failed when scats were more than 54 h old. Failed reactions, however, did not increase with storage time when total DNA concentration exceeded 5 ng/ μ L (0.5% for isolates from scats <54 h old and 0.0% from scats >54 h old). DNA yield was, however, significantly lower from stored scats relative to scats <30 h old, so isolating DNA from fresh scats will maximize DNA yield and thus increase genotyping reliability.

Sexing markers—DNA isolated from four male koalas and five female koalas was successfully amplified using Y- and X-linked sexing markers. The sex of the individual sampled was correctly designated in all cases (Fig. 2). These markers may be combined with the microsatellite suite to obtain both the sex and unique genotype of the individual sampled.

Discussion

Genetic sampling of wild, living koalas can be used to provide data that are currently lacking for most koala populations (EaCRC 2011). We have demonstrated that DNA recovered by surface washing of a single koala scat provides a reliable individual genotype and may therefore be used to identify individual koalas and be used for population genetic studies. Obtaining DNA isolates of sufficient quantity and quality from a single scat negates the need to isolate DNA from multiple pellets, as is sometimes required (e.g. Soto-Calderon *et al.* 2009), thus minimizing the chance of sample contamination between different individuals. Screening DNA isolates for total DNA concentration allows inferior isolates (<0.25 ng/ μ L) to be discarded prior to genotyping and samples with sufficient amounts of DNA to be analysed using the minimum number of required replicates, thereby minimizing costs. A summary of the methods developed in this study for reliable DNA isolation and genotyping from a single koala scat is provided in Fig. 3; by following the recommendations determined in this study, optimal quality DNA isolates should be obtained.

DNA degradation is increased when biological samples are exposed to moisture (Piggott 2004). This

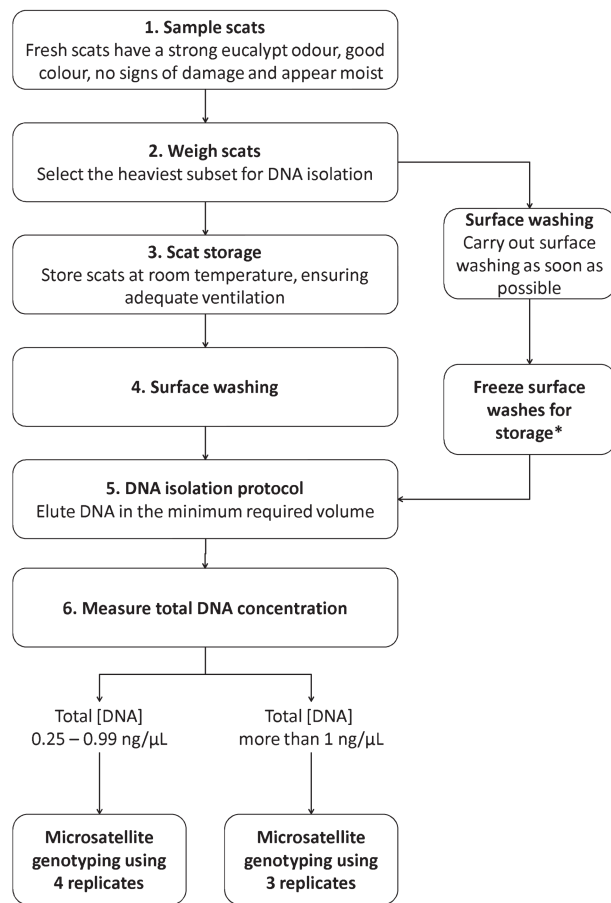


Fig. 3 Flowchart illustrating methods used to reliably genotype koalas from scat samples. [DNA]: DNA concentration, * Alternate untested storage method that may increase DNA yield.

study demonstrated that scats exposed to inclement weather had higher amplification failure, allelic dropout and false alleles, compared to sheltered samples. Brinkman *et al.* (2010) also observed high amplification rates in deer pellets protected from rain over 4 weeks, compared to those exposed to weather. In the absence of rain, or where scats are found in weather-protected positions, field collection of samples up to 4 weeks old should produce DNA isolates that can be reliably used for genotyping studies. Collection of scats within this time frame should be easily achieved. Scats <2 weeks old can be recognized in the field by the presence of a shiny coating on the scat, the absence of cracking and a distinctive eucalypt odour (Sullivan *et al.* 2002). A single koala can produce up to 200 scats per day (Ellis *et al.* 1999) and is not likely to have been residing in the same tree for more than 24 h (Ellis *et al.* 2009). It has also been shown that 50% of total scat production is expected to be located within 1 m of the tree base (Phillips & Callaghan 2011), making finding samples an efficient process, especially if a koala is present which would attract a searcher to a tree.

While storage of scats in paper bags has been found to be effective for the brush-tailed rock wallaby (*Petrogale penicillata*), the red fox (*Vulpes vulpes*) and the northern hairy-nosed wombat (*Lasiorhinus krefftii*) (Piggott & Taylor 2003a; Walker *et al.* 2009), this study found that genotyping reliability was significantly lower for scats stored in paper bags compared to those left outside in a sheltered position. Volatile substances evaporating from the scat may be retained within the paper bag, thereby inducing cell and DNA damage. Volatile molecules, phenolics or microbial contaminants may also be carried over into the surface wash and DNA isolates, potentially inhibiting subsequent amplification and decreasing genotyping reliability.

Samples stored in the open for up to 4 weeks were shown to have low rates of genotyping error. The amount of DNA isolated was, however, significantly lower from stored scats, potentially due to decreased surface washing efficiency for dry scats. Obtaining lower DNA yields will necessitate a greater number of replicates, therefore increasing genotyping costs. DNA is therefore ideally isolated from a fresh scat sample, although this may not always be possible. Faecal samples are commonly stored at -20°C to prevent further sample degradation (Ball *et al.* 2007; Spiering *et al.* 2009). Liquid nitrogen storage of surface washes carried out in the field for the northern hairy-nosed wombat has been found to produce DNA isolates with low levels of amplification failure and genotyping errors (Walker *et al.* 2009). Freezing surface washes may therefore provide an alternative storage method that could increase the yield of DNA isolated from koala scats (Fig. 3).

Genetic sampling of a koala from a single scat can provide DNA that is suitable for microsatellite genotyping and a range of ecological investigations. Obtaining genetic samples from scats will enable the collection of large sample sizes, without introducing sampling bias or interfering with the study animals. Population data may subsequently provide information that could help to prioritize and inform management strategies relating to koala conservation in Australia, aiding the long-term viability of this species.

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All authors contributed to the designed of the research. F.W. and M.K. performed the laboratory work and data analysis. F.W., F.H. and J.M. wrote the manuscript.

Data Accessibility

DNA concentrations, microsatellite data, GIMLET and GEMINI input files: DRYAD entry doi: 10.5061/dryad.n2m8v.

Chapter 3 | foreword

Chapter two described the identification of appropriate collection and storage methods for koala scats, and also determined the number of microsatellite genotype replicates required to ensure reliable consensus genotypes. Chapter two used replicate genotyping to estimate error rates and simulated data to determine the number of replicates required to obtain reliable data. Some errors, such as null alleles, are highly repeatable and can, therefore, remain undetected using replicate genotyping. The purpose of chapter three was, therefore, to validate that the methods devised in chapter two, would in fact, provide accurate genotype data.

In chapter three, scats were sampled from koalas within a captive colony, where relationships between individuals were mostly known. Genetic data and pedigree data could then be compared, and errors in consensus genotypes identified by checking for Mendelian inheritance between parents and their offspring. This allowed scoring methods and the number of replicates, determined in chapter two, to be validated.

Genotypes can unequivocally discriminate between individuals; the statistical likelihood that two individuals share identical genotypes by chance can be estimated using the probability of identity (P_{ID}) and probability of identity between siblings (P_{IDsibs}). Access to pedigree data relating to the captive population studied in chapter three also facilitated empirical determination of the power of the chosen microsatellite suite to discriminate between individuals, including close relatives, by examining the number of loci with genotype differences between pairs of individuals.



Chapter 3

Validating the use of non-invasively sourced DNA for population genetic studies using pedigree data



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Validating the use of non-invasively sourced DNA for population genetic studies using pedigree data

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Abstract. Non-invasive genetic sampling has provided valuable ecological data for many species – data which may have been unobtainable using invasive sampling methods. However, DNA obtained non-invasively may be prone to increased levels of amplification failure and genotyping error.

Utilizing genotype data from 32 pedigreed koalas, this study aimed to validate the reliability of final consensus genotypes obtained using DNA isolated from koala scats. Pedigree analysis, duplicate genotyping, analysis of mismatched loci and tests for null alleles were used to look for evidence of errors.

All genetically confirmed parent–offspring relationships were found to follow Mendelian rules of inheritance. Duplicate genotypes matched in all cases and there was no evidence of null alleles. Related individuals always had different 12-marker genotypes having a minimum of three unique loci (in one full sibling pair), a mode of seven unique loci and a maximum of 11 unique loci.

This study demonstrates the capacity of DNA recovered from koala scats to provide reliable genotypes that can unequivocally discriminate individuals and infer parentage, provided data are missing from no more than two loci. Validating data obtained using non-invasive sampling is an important step, allowing potential problems to be identified at an early stage.

1 Introduction

Koalas (*Phascolarctos cinereus*) are elusive and typically reside in tall eucalypt trees, which can make animal capture and the collection of samples (e.g. blood or biopsy) for DNA analysis costly and time consuming. Koala scats are a convenient DNA source that can be readily identified and collected from the forest floor. DNA isolated from koala scats can then be used to genotype individual koalas, providing a unique DNA profile including the gender of the individual sampled (Wedrowicz et al., 2013). The genotypic data obtained can also be used for a range of population genetic analyses. Previous genetic studies of koala populations have used DNA recovered from blood or biopsy samples to study koala populations using microsatellites (Houlden et al., 1996; Lee et al., 2011) and mitochondrial control region sequencing (Houlden et al., 1999). Sourcing DNA non-invasively from scats offers

a valuable tool that may be useful for genetic studies of wild populations (Morin et al., 2016; Piggott et al., 2006; Stenglein et al., 2011). The ability to identify individuals using molecular methods is critical for determining the number of unique individuals sampled. This information is also important for a range of other applications such as population monitoring and estimating population size using mark–recapture methods as well as investigating social structure, relatedness and dispersal (Taberlet et al., 1997). However, the presence of errors in consensus genotypes may incorrectly result in genotypes from the same individual appearing as though two different individuals have been sampled. This type of error may bias analyses, resulting in overestimation of the number of individuals sampled and thus population size (Waits et al., 2001), which may in turn reduce the reliability of inferences.

DNA samples obtained using non-invasive collection methods are often associated with greater rates of amplifica-

tion failure and genotyping error compared to those obtained from invasively collected samples such as from tissues (Pompanon et al., 2005; Taberlet et al., 1996). The major factors contributing to increased amplification failure and error rates in non-invasively collected samples are DNA degradation (reducing DNA quality) and lower quantities of DNA due to collection of fewer cells from non-invasive sources (Taberlet et al., 1996). Genotyping errors are noted when two or more genotypes appear to have originated independently from the same sample, the major error types being allelic dropout and false alleles (Beja-Pereira et al., 2009; Bonin et al., 2004). Allelic dropout (ADO) occurs when only one of the two alleles in a heterozygote is amplified, while false alleles (FA) are amplified PCR artefacts that may be mistaken for a true allele (Pompanon et al., 2005). Though there is always likely to be some degree of error in any given data set, the increased chance of errors in DNA collected from non-invasive sources necessitates the assessment and minimization of errors.

A method for the isolation and microsatellite genotyping of koala DNA from scats has been described by Wedrowicz et al. (2013) and levels of error were reported to be 1.8 % (ADO) and 0.3 % (FA) when DNA concentration was above $1 \text{ ng } \mu\text{L}^{-1}$. Based on simulations, and depending on DNA concentration, three or four genotyping replicates were required to provide highly reliable genotype data. Consensus genotypes constructed from replicates were, however, not validated. The availability of empirical data permits the presence of errors in consensus genotypes to be examined and allows the likelihood that genotypes from two different individuals are the same or similar to be estimated (Paetkau, 2003).

Here we consider genotypic data obtained using DNA collected from a captive and pedigreed koala population to validate a non-invasive method for DNA collection and genotyping (Wedrowicz et al., 2013). The aim of this study is to confirm whether the number of replicate genotypes and scoring rules used by Wedrowicz et al. (2013) produce correct consensus genotypes for individual koalas.

2 Materials and methods

2.1 The captive koala population

The Koala Conservation Centre (KCC) located on Phillip Island, Victoria, Australia, began operation in 1992 in response to concerns regarding the long-term survival of Victorian koala populations, including those on Phillip Island. The KCC comprises approximately 7.6 ha of forest (enclosed and subdivided by koala proof fences) where three tree species browsed by koalas dominate: southern blue gum (*Eucalyptus globulus*), manna gum (*E. viminalis*) and swamp gum (*E. ovata*). The KCC currently sustains a free-ranging population of around 40 koalas, some of which are restricted to particular areas of the property within large internally fenced areas. The centre's koala population was initially established

using individuals from the South Gippsland region in Victoria, due to their presumed endemicity and low rates of symptomatic chlamydial disease despite a high prevalence of infection (Emmins, 1996).

2.2 Scat collection and DNA isolation

Three fresh scat samples (showing a shiny outer surface) were collected from the ground directly beneath 32 individual koalas of known identity on two sampling occasions during 2007 ($n = 11$) and 2013 ($n = 21$). Samples collected in 2007 were independent from those used in the Wedrowicz et al. (2013) study. Individual koalas were identified by colour-coded ear tags. Scats were collected using wooden toothpicks and stored upright in open ended containers until surface washing. DNA was retrieved from a surface wash of each scat as described in Wedrowicz et al. (2013). To prevent sample contamination, DNA isolation, PCR setup and post-PCR analysis were carried out in separate areas of the laboratory and filtered pipette tips were used for pipetting DNA or DNA products. DNA was isolated from surface washes of two of the three scat samples for each individual animal using the QIAamp® DNA stool mini kit (Qiagen) as described by Wedrowicz et al. (2013); the surface wash of the third scat was stored in reserve at -20°C . DNA isolation from samples collected in 2007 used a slightly different protocol to that described in Wedrowicz et al. (2013) as outlined in the Supplement.

2.3 DNA screening and genotyping

DNA was quantified using the Qubit® dsDNA HS assay kit (Life Technologies). DNA quality was assessed using standard PCR and electrophoresis. Microsatellite Pcv31 (Cristescu et al., 2009) was amplified using reactions comprising $5 \mu\text{L}$ GoTaq® green master mix (Promega), $0.5 \mu\text{M}$ of each primer and $0.1 \mu\text{g } \mu\text{L}^{-1}$ of bovine serum albumin (BSA) made up to $10 \mu\text{L}$ with nuclease free water. A sexing PCR using primers GpdEx12, GpdEx13R (Loebel et al., 1995; Loebel and Johnston, 1997), IMY1 and IMY2 (Watson et al., 1998) and the PCR parameters described in Wedrowicz et al. (2013) was also carried out. For each individual, the DNA isolate producing the brightest bands on PCR gels, was chosen for genotyping.

Wedrowicz et al. (2013) describes a method for reliably genotyping koalas from DNA isolated from a single koala scat using specific sample collection and storage procedures and optimized DNA isolation protocols. Genotyping errors are accounted for by replicating genotypes three or four times per sample, according to DNA concentration, minimizing the chance that final genotypes contain errors. Replicate genotypes are used to produce a consensus genotype, with more than 99 % confidence in the resultant genotype (Wedrowicz et al., 2013). However reproducible errors, such as null alleles, may remain undetected using this method, requiring

other forms of error checking such as pedigree analysis for detection (Pompanon et al., 2005).

To obtain genotypes for each sample, DNA isolates were amplified using 12 microsatellite markers: K2.1, K10.1, Pcv2, Pcv6.1, Pcv6.3, Pcv24.2, Pcv25.2, Pcv30, Pcv31 (Cristescu et al., 2009), Phc2, Phc4 and Phc13 (Houlden et al., 1996). Amplification and product separation using capillary electrophoresis was conducted at the Australian Genome Research Facility (AGRF), Melbourne, Australia. Genotypes were replicated as recommended by Wedrowicz et al. (2013) according to the total DNA concentration (three replicates if DNA concentration was greater than $1.0 \text{ ng } \mu\text{L}^{-1}$; four replicates if DNA concentration was $0.25\text{--}1.0 \text{ ng } \mu\text{L}^{-1}$).

2.4 Estimation of error rates

Rates and instances of genotyping error were calculated from replicate genotypes using GIMLET v 1.3.3 (Valière, 2002). Consensus genotypes were constructed based on Taberlet et al. (1996) and Valière et al. (2007) using the following rules: (1) alleles had to appear at least twice to be counted; (2) where four replicates were used, homozygous alleles had to appear at least three times; and (3) loci giving ambiguous results were omitted (scored as a failed reaction). In order to check for genotyping errors between consensus genotypes of independent duplicates (different scats from the same individual), DNA isolated from the second scat was also genotyped for nine randomly chosen individuals (28 % of samples). Micro-checker was used to test for the presence of null alleles (Van Oosterhout et al., 2004).

2.5 Mitochondrial DNA sequencing

To obtain maternally inherited haplotype data for the KCC koala population, primers KmtL1 and KmtH2 (Fowler et al., 2000) were used to amplify and sequence approximately 700 base pairs within the mitochondrial DNA (mtDNA) control region. Mitochondrial DNA was amplified using BIO-X-ACT™ short DNA polymerase (Bioline). Reactions consisted of $1 \times$ OptiBuffer, $1 \times$ Hi-Spec additive, 2 mM MgCl_2 , 0.5 mM each dNTP, 1 unit of BIO-X-ACT™ short DNA polymerase and $0.5 \mu\text{M}$ of each primer, made up to $20 \mu\text{L}$ with water. DNA was initially denatured for 3 mins at 95°C , followed by 35 cycles of 94, 50 and 72°C for 1 min each and finishing with a single final extension of 5 min at 72°C . PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequencing was carried out by AGRF. Resulting sequences were aligned and trimmed using MEGA 6 (Tamura et al., 2013).

2.6 KCC pedigree data

Pedigree information was obtained from KCC records. Five maternal founders and five generations were represented in the KCC pedigree (Fig. 1). At the Conservation Centre,

koalas within the same maternal lineage are generally given names that begin with the same letter; names assigned by the KCC to each individual are used throughout this paper. Maternal relationships were known with high confidence as juveniles were caught and tagged after leaving the pouch but before becoming independent. Confident identification of paternal relationships can be more challenging as, although individual female–male pairs are housed together within the same enclosure for breeding, other KCC koalas may sometimes escape or enter the enclosures; opportunities for other males within the captive colony to breed with the intended female therefore exist. To account for potential errors in the pedigree, all parental relationships were considered putative until confirmed by the molecular data. The term “putative” is therefore used throughout when referring to the KCC pedigree data alone.

2.7 Probability of identity

The probability of identity and probability of identity between siblings were calculated using GenAlEx (Peakall and Smouse, 2012). The similarity between genotypes of pairs of individuals was also considered by examining the number of loci with different genotypes as described by Paetkau (2003). We use the same system as Paetkau (2003) to describe the number of mismatched loci between pairs of individuals. Two individuals with identical genotypes at all 12 loci (no mismatches) are referred to as a 0MM pair; a 1MM pair describes a pair of individuals with a single mismatching locus, i.e. a unique genotype at one locus and identical genotypes at all other loci; and a 12MM pair has a unique genotype at every locus (Paetkau, 2003). To count the number of mismatched loci between each pair of individuals in the KCC population, the R package *allelematch* (Galpern et al., 2012; R Core Team, 2014) was used.

In order for the degree of relatedness to be compared to the frequency of mismatched loci, parental information from the KCC pedigree confirmed by the genetic data was used to calculate pedigree relationship coefficients (R) between all pairs of individuals using the *pedantics* package (Morrissey and Wilson, 2010; R Core Team, 2014). Parentage information was omitted where the parents of an individual koala were unknown or uncertain, meaning that a small number of pairwise relationships may have been classified as unrelated when the true degree of relationship may have been higher. Values calculated by the *pedantics* package (derived from the genetically confirmed pedigree data) were used to assign each pairwise relationship as first order (I: parent–offspring (PO) or full siblings (FS), $R \geq 0.50$), second order (II: half-siblings (HS), avuncular or grandparent–grandoffspring, $0.25 \leq R < 0.50$), third order (III: cousins, $0.125 \leq R < 0.25$) or unrelated (UR: $R < 0.125$).

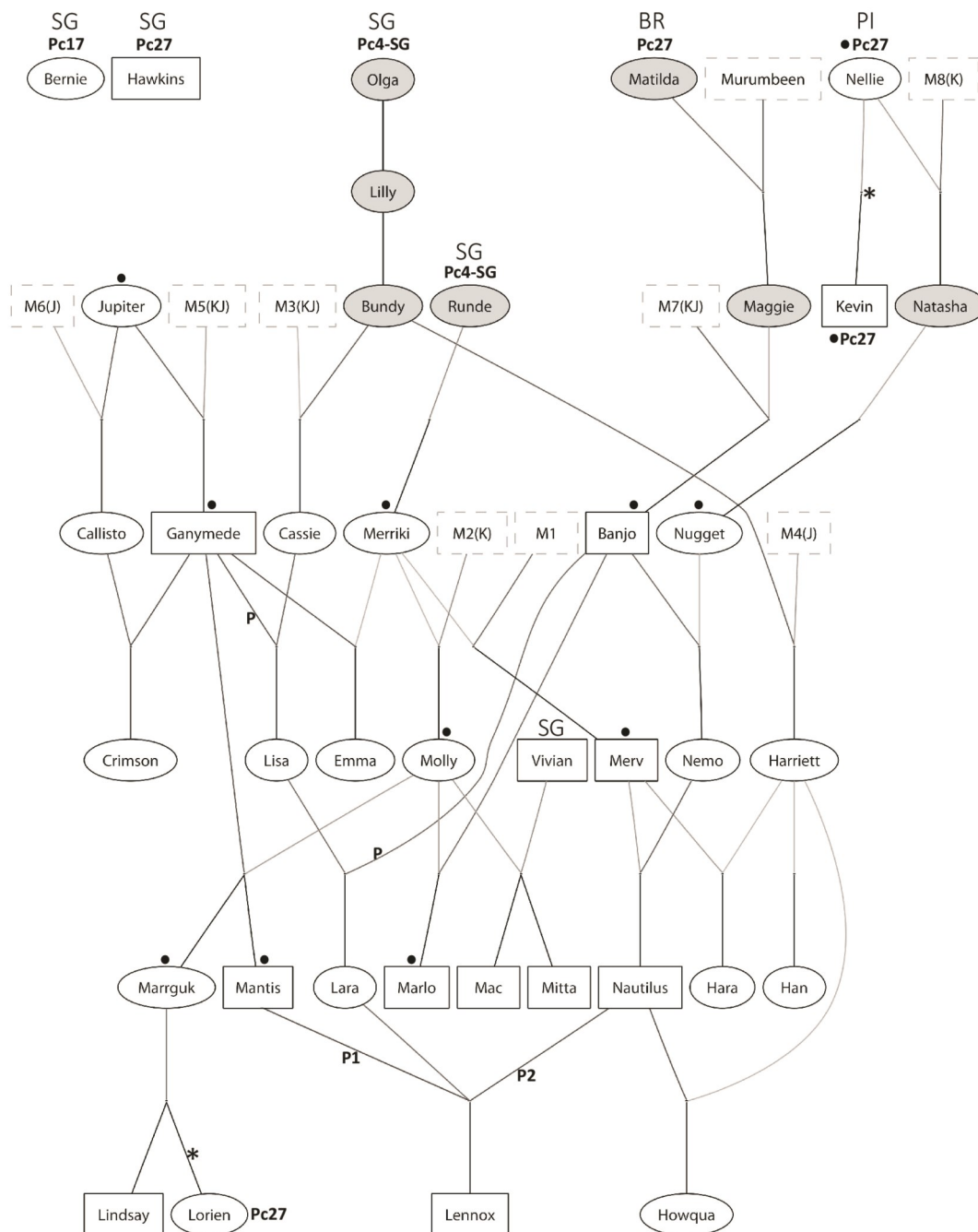


Figure 1. Pedigree diagram for sampled koalas. Ovals indicate females and rectangles males. Individuals marked with a circle (●) were sampled in 2007, while all others were sampled in 2013. Molly and Marlo were sampled in both 2007 and 2013. Individuals that are shaded grey were not sampled. Presumed maternal relationships not supported by the molecular data are marked with an asterisk (*). Putative paternal relationships not confirmed by the genetic data are not shown. Possible paternal relationships identified from the molecular data are marked with a “P” or “P1” and “P2” where more than one candidate father was identified. The origin of founders is also indicated by SG: South Gippsland; PI: Phillip Island; or BR: Brisbane ranges (founded by French and Phillip Island stock). Unknown fathers are numbered M1–M8. Many of these fathers are thought to be Karlos (K), Jack (J) or one of the two (KJ), though these individuals were not sampled.

Table 1. Genotyping success rates according to sampling year and DNA concentration, grouped into categories A–D.

Total DNA isolated (ng μL^{-1})	Sampling year	Total PCRs (no. of individuals)	PCR success rate ($\pm 95\%$ CI %)	Average peak height for successful PCRs ($\pm 95\%$ CI)	Average number successful loci per consensus genotype	Allelic dropout: average rate across loci % (frequency)	False alleles: average rate across loci % (frequency)
A: 0.10–0.25	2007	96 (2)	90 \pm 4.5	7591 \pm 1098	11.0/12	1.0 (1)	0.0 (0)
	2013	48 (1)	71 \pm 6.6	4236 \pm 610	8.0/12	0.0 (0)	0.0 (0)
	Overall	144 (3)	80 \pm 4.1	6110 \pm 693	10.0/12	1.0 (1)	0.0 (0)
B: 0.25–0.50	2007	192 (4)	69 \pm 3.3	4665 \pm 437	8.0/12	5.6 (5)	0.0 (0)
	2013	192 (4)	89 \pm 2.3 ^A	5641 \pm 405	11.3/12	5.1 (5)	0.0 (0)
	Overall	384 (8)	79 \pm 2.1 ^A	5214 \pm 298	9.6/12	6.2 (10)	0.0 (0)
C: 0.50–1.0	2007	0 (0)	–	–	–	–	–
	2013	288 (6)	92 \pm 1.6	5420 \pm 328	11.8/12	0.8 (2)	0.8 (2)
	Overall	288 (6)	92 \pm 1.6 ^{A,B}	5420 \pm 328	11.8/12	0.8 (2)	0.8 (2)
D: > 1.0	2007	252 (7)	88 \pm 1.9 ^{A,B}	5040 \pm 317	10.6/12	2.7 (5)	0.8 (1)
	2013	648 (18)	97 \pm 0.65 ^C	7539 \pm 265 ^{B,C}	11.8/12	0.4 (2)	0.0 (0)
	Overall	900 (25)	94 \pm 0.76 ^{A,B}	6812 \pm 212 ^{B,C}	11.4/12	1.1 (7)	0.2 (1)

^{A, B, C} Superscripts indicate categories that differed significantly ($p < 0.05$).

2.8 Parentage analysis

The full exclusion method of paternal analysis was carried out manually to confirm parent–offspring relationships. Offspring genotypes were considered compatible with parental genotypes if one allele was shared at each of the 12 loci genotyped. PARENTE (Cercueil et al., 2002) uses the same method to identify potential mothers, fathers and parent pairs and was also used to confirm parentage. Given known mothers, tests for paternity were carried out using CERVUS (Kalinowski et al., 2007). KCC records and the genotyping results were used to visualize the KCC pedigree sampled using Pedigraph V1 (Garbe and Da, 2008; Fig. 1). Parentage was also determined from the genotypic data using the pedigree reconstruction software FRANz 2.0.0 (Riester et al., 2009). FRANz was utilized as it is able to infer multigenerational pedigrees without the need for prior information, such as maternal relationship, that is often required by other software.

3 Results

3.1 Genotypic data

Consensus DNA profiles were obtained for 32 koalas at the KCC. Genotypic data were obtained for a total of 42 DNA isolates (32 individuals: 23 single samples, 9 duplicate samples and 1 triplicate sample); of these, 17 were genotyped four times (DNA concentration below 1 ng μL^{-1}) and 25 were genotyped three times (DNA concentration over 1 ng μL^{-1}). Of the 32 individuals genotyped, 21 (66 %) had complete genotypes, 3 had missing data at one locus (9 %), 4 had missing data at two loci (12 %) and 4 individuals (9 %)

were not successfully amplified or scored at more than two loci (three, four, four and seven missing loci). Missing data were mostly present in samples collected in 2007 (Table 1), which could be due to the slightly different method used for DNA isolation. All consensus genotypes were identical at every available locus for the scat samples from the nine individuals genotyped in replicate (Supplement, Table S1). Scats from two individuals (Molly and Marlo) were obtained in both 2007 and 2013; for each individual, genotypes at all available loci were identical for the 2007 and 2013 samples (Supplement, Table S1), confirming that the intended individual was sampled on both occasions.

The rates of allelic dropout and false alleles calculated from replicate data were 1.9 and 0.2 % (averaged across loci) respectively, which is similar to the overall error rates of 1.8 and 0.3 % reported by Wedrowicz et al. (2013) for samples containing 1–20 ng μL^{-1} of total DNA. No evidence of errors in consensus genotypes obtained from the KCC population were noted. Five putative parent–offspring relationships (two maternal and three paternal) were found to be incompatible. Incompatible genotypes identified in putative parent–offspring relationships displayed (1) mismatched genotypes at multiple loci and (2) unanimity with additional known relationships and so were refuted as errors. Four of the five incompatible parent–offspring pairs had more than three mismatching loci. One putative parent pair (Lara–Merv) mismatched at only one locus (K2.1), where both the parent and offspring were heterozygous. In the parent (Merv), the discrepant allele of 166 base pairs was refuted as an error due to its presence in his mother and another of his offspring (Supplement, Fig. S1). The mismatching 164 base pair allele

Table 2. Variability in 640 bp mtDNA control region sequence for individuals sampled at the KCC.

Haplotype	<i>n</i>	Percent of samples	Nucleotide position			
			6	21	112	247
Pc27	13	40.6 %	G	T	G	G
Pc4-SG	18	56.3 %	–	–	–	A
Pc17	1	3.1 %	A	C	A	A

was present in two independent samples from the offspring (Lara), and was present in seven other individuals within the population; CERVUS identified another candidate male (Banjo) as the most likely father. Mutation of the K2.1 allele from 166 bases in the parent (Merv) to 164 bases in the offspring (Lara) is also a possibility though the mutation rate of microsatellites, averages around 5×10^{-4} mutations per locus per generation (Selkoe and Toonen, 2006). Further genotyping using additional markers for Lara (offspring), Lisa (mother) and Merv and Banjo (potential fathers) would therefore be required to definitely assign parentage in this case. All genotypes were found to follow Mendelian modes of inheritance. Null alleles were not detected using Micro-Checker. Considering all individuals sampled at the KCC (including Bernie and Hawkins; two recent immigrants introduced from South Gippsland), Hardy–Weinberg proportions (HWP) were followed for all but one locus (Pcv6.3); all loci were in HWP after the two immigrants, which had no sampled offspring, were removed.

3.2 Sequencing performance and mtDNA variability

Following alignment and trimming, 642 bases of mtDNA control region sequence were obtained. Three mtDNA haplotypes with four variable sites were identified in the KCC individuals sampled in this study (Table 2). Haplotype names have recently been standardized by Neaves et al. (2016) and are used here. Two of the haplotypes, Pc17 and Pc27, were previously described by Houlden et al. (1999). Pc27 is the most common haplotype in South Gippsland and the only haplotype found in individuals of French and Phillip Island origin (Houlden et al., 1999); haplotype Pc27 was present in 13 (41 %) of the 32 KCC individuals. One (3 %) immigrant koala (Bernie) possessed the Pc17 haplotype. The third haplotype, designated Pcv4-SG, has not been previously reported in Victoria. Pcv4-SG was the most common haplotype in the KCC data set, being present in 18 (56 %) of the 32 individuals sampled. Pcv4-SG was 100 % identical to Pc4 reported by Houlden et al. (1999) found in northern NSW koalas (Coonabarabran and Port Stephens) but only covered 74 % of the full length of Pc4. Sequencing a larger section of the control region would be required in order to more accurately define this haplotype.

Table 3. The number of loci displaying different genotypes between pairs of individuals of known relationship in the KCC koala population. FOR: first-order relationship ($R \geq 0.50$); SOR: second-order relationships ($0.25 \geq R > 0.50$); TOR: third-order relationship ($0.125 \geq R > 0.25$); UR: unrelated ($R < 0.125$).

Mis-matched loci	FOR	SOR	TOR	UR	Total (percent related)
0MM	0	0	0	0	0 (–)
1MM	0	0	0	0	0 (–)
2MM	0	0	0	0	0 (–)
3MM	1	0	0	0	1 (100 %)
4MM	1	2	0	3	6 (50 %)
5MM	4	2	2	8	16 (50 %)
6MM	0	8	2	19	29 (34 %)
7MM	8	6	8	51	73 (30 %)
8MM	5	6	8	68	87 (22 %)
9MM	2	7	6	70	85 (18 %)
10MM	1	3	4	37	45 (18 %)
11MM	0	0	1	26	27 (4 %)
12MM	0	0	0	9	9 (0 %)
Totals	22	34	31	291	378 (23 %)

3.3 Individual identification

All positive sexing PCRs correctly identified the gender of the individual sampled. Using the complete 12-marker set of microsatellites, the probability of identity (P_{ID}) and probability of identity between siblings ($P_{ID\text{sibs}}$) in the KCC population was 1.3×10^{-8} (≈ 1 in 75 000 000) and 3.9×10^{-4} (≈ 1 in 2500 or 0.04 %), respectively. To retain a high level of power of the marker set to discriminate individuals, a limit of missing data at two loci was imposed (i.e. consensus genotypes with missing data at more than two loci were discarded). The P_{ID} and $P_{ID\text{sibs}}$ in the KCC population using the ten least informative loci (i.e. excluding the two most informative loci: K2.1 and Pcv2) was 2.5×10^{-6} (≈ 1 in 400 000) and 2.8×10^{-3} (≈ 1 in 350 or 0.28 %). Excluding consensus genotypes with missing data at more than two loci, there were no instances of 0MM, 1MM or 2MM pairs. There was one occurrence of a 3MM pair between full siblings (Mitta and Mac) and six 4MM pairs (three related and three unrelated: Table 3), suggesting that the likelihood of any two individuals having the same 12-marker genotype is likely to be very low. Using this microsatellite suite for the individual identification of koalas at the KCC, we therefore recommend that at least ten loci are successfully typed and scored to have a high level of confidence in individual assignment.

3.4 Parentage analysis

A pedigree diagram was constructed for the individuals sampled in this study using the KCC pedigree data, supplemented by information obtained from the molecular data (Fig. 1).

The pedigree information supplied by the KCC consisted of 22 putative maternal relationships and 14 putative parent pair–offspring relationships. Of the mother–offspring relationships 20 of the 22 (91 %) were confirmed using the full exclusion method. Two individuals (Kevin and Lorian) were not tagged when juveniles; hence, there was some initial uncertainty regarding their parentage. The putative maternal relationships for Kevin and Lorian were not confirmed by the genetic data (Kevin and Lorian had four and three mismatched loci with their presumed mothers respectively). Genotypic data identified Jupiter as a potential mother of Kevin, with a matching allele at each of the 10 loci available between the pair. None of the sampled individuals were found to have a genotype compatible with maternity of Lorian; however, not all potential parents at the KCC were sampled. As expected, all mtDNA haplotypes were maternally inherited. Haplotype data also contradicted the parent–offspring relationship between Marrguk (Pc4–SG) and Lorian (Pc27), showing that Lorian originates from a maternal lineage with the Pc27 haplotype (Fig. 1).

Putative paternal relationships (and therefore parent pairs) were confirmed for 11 out of 14 (78 %) offspring, while three putative paternal relationships (the putative fathers of Lisa, Lara and Lennox) were refuted by the genotypic data. For the three individuals whose presumed fathers were not validated by the molecular data (Lisa, Lara and Lennox, Fig. 1), alternative potential fathers were identified from the males sampled. Since all males at the KCC were not sampled these relationships could not be unequivocally confirmed; however, paternity assignment using CERVUS specified significant odds ratios for the Ganymede–Lisa, Banjo–Lara and Mantis–Lennox father–offspring relationships.

3.5 Pedigree reconstruction

The pedigree reconstruction software, FRANz 2.0.0 (Riester et al., 2009) was used in order to evaluate whether a multigenerational pedigree could be constructed based on the genetic data (12-marker genotypes and sex markers) alone. Individuals with missing data at more than two loci were excluded, leaving nine maternal relationships and seven parent pairs with genetically confirmed relationships. All nine maternal relationships and seven parent pairs were correctly identified by FRANz. Additionally, the known mothers/potential fathers for two of the three individuals with uncertain paternity were also identified by FRANz. All identified parent pairs had a log of the odds (LOD) value greater than 4.0, meaning that the odds of the identified relationships detected having occurred by chance are greater than 10^4 to 1. One individual (Lindsay; 7.7 % of identifications above $\text{LOD} = 4$) was incorrectly assigned to a parent pair by FRANz. Since Lindsay's true mother amplified at only five loci and was excluded, the true mother's full sibling was assigned instead; the true father was unknown. A LOD of 4 will therefore provide a good cutoff above which parents identi-

fied using FRANz can be considered highly likely, provided genotypes do not have missing data at more than two loci.

4 Discussion

Declining wildlife populations and threatened or vulnerable species may benefit from the development of non-invasive genetic sampling regimes allowing for the rapid acquisition of large amounts of population data. Such data have the potential to facilitate a greater understanding of the processes that may be implicated in population declines and thus allow for the development and action of appropriate management strategies in an attempt to prevent further declines and potential extinction. By sampling scats from a captive population of koalas, with known pedigree, this study has shown that the method for isolating and genotyping DNA from koala scats used here provides accurate consensus genotypes. This study also demonstrated the ability of the 12-microsatellite suite to unequivocally identify the individual from which the sample was obtained and to infer parent–offspring relationships. The applicability of mtDNA sequencing using DNA isolated from koala scats was also established. Overall, this study has confirmed that sampling DNA from koala scats is a robust and reliable alternative to traditional DNA sources that may be beneficial to future conservation studies for the koala.

4.1 Error assessment

Similar to results reported by Wedrowicz et al. (2013), the error rate found within replicate genotypes was determined to have an average of 1.9 % (ADO) and 0.2 % (FA) across loci. Using pedigree analysis, duplicate genotyping, analysis of mismatched loci and tests for null alleles, the consensus genotypes of the 32 individuals used in this study were free of detectable error. This provides a strong indication that the methods and scoring rules used to obtain genotypic data using DNA isolated from koala scats are associated with acceptably low error rates.

Examination of pairs of individuals with a small number of mismatched alleles (Paetkau, 2003) can be a very useful method for checking errors in samples collected from wild populations. Within the captive KCC population, no 0MM (same 12-marker genotype), 1MM or 2MM pairs were observed, while a 3MM pair was observed once between a pair of full siblings. Given that the KCC population is likely to contain a higher proportion of related individuals than wild populations, it would seem unlikely that any two individuals would have less than four mismatched loci between them. Identifying genotypes that mismatch at three or less loci in wild collected data sets is an ideal step for identification of potential errors before carrying out further analyses.

4.2 Individual identification

The suite of markers considered here can be used to unequivocally identify individuals and infer parentage, which are both important capabilities for ecological investigations of koala populations. As discussed above, the distribution of mismatched loci found between pairs of individuals in the KCC population, containing numerous first degree relatives, suggests that the chance of observing a 0MM pair is extremely small. P_{ID} and P_{IDsibs} for the koala population at the KCC was 1 in 75 000 000 and 1 in 2500 when all 12 markers were successfully amplified. P_{IDsibs} is suggested as a conservative limit from which to gauge the probability of two individuals sharing the same genotype by chance (Waits et al., 2001). The frequency of full siblings in wild koala populations is unknown, but likely to be negligible as most siblings born to the same mother are found to have differing paternities (Ellis et al., 2002). P_{IDsibs} is therefore a very conservative measure for defining genotype matches for koalas, which would be advantageous as incorrectly identifying individuals could lead to misinformed management. Previously utilized cut-offs for confident assignment of full genotype matches as one specific individual have included 0.05 (1 in 20; Woods et al., 1999) for P_{IDsibs} and < 0.001 – 0.0001 (less than 1 in 1000–10 000) P_{ID} in wildlife forensics (Waits et al., 2001). The P_{ID} and P_{IDsibs} reported in this study are therefore well within acceptable limits.

4.3 Inference of parentage

Genetic data can be used to assist captive management in a range of ways, including assessments of founder relationships, filling gaps within pedigrees, quantifying and monitoring genetic diversity, classifying the region of origin and identifying genetically valuable individuals (Ivy et al., 2009). Parent–offspring relationships were confirmed in almost all cases in this study (31/36). Two uncertain maternal relationships not confirmed by the genetic data highlights the potential for errors to be present in pedigree information. Pedigree data are often used in captive breeding programmes to select breeding pairs that are sufficiently unrelated while genetic estimates of relatedness can be useful to minimize inbreeding between wild founders (Bergner et al., 2014). When using pedigree data, the presence of pedigree errors may undermine management schemes, resulting in unintended breeding between related individuals that could have a negative effect on fitness (Hammerly et al., 2016). Supplementing pedigree data with genetic data may be useful for captive management programmes in order to ensure management decisions are based on the most accurate information possible by using genetic data to validate pedigree data and chosen breeding pairs. As an example, a study of captive Attwater’s prairie chickens carried out by Hammerly et al. (2016) firstly assigned breeding pairs based on pedigree estimates of relatedness and then reassigned the chosen pair if the genetic es-

timate of pairwise relatedness was greater than 0.125. This method of mate selection was found to significantly increase the survival rate of chicks compared to choosing breeding pairs based on the pedigree alone (Hammerly et al., 2016). Inferring parentage within wild populations with no prior pedigree knowledge is also possible, though likely to present a greater challenge, as sampling of all candidate parents (especially fathers) may not always be achievable.

5 Conclusions

DNA isolated from koala scats, and investigated using a suite of DNA markers, provided data that can be confidently used to study both captive pedigrees and wild koala populations. Analyses of parentage and relatedness aid the selection of breeding pairs and can verify the parents of juveniles when parental information is unavailable or uncertain, so can assist the management of captive populations. The use of non-invasive genetic sampling for the study of wild populations has the potential to provide numerous advantages over invasive sampling methods. Compared to obtaining DNA from biopsies or blood from wild individuals, sourcing DNA from scats confers the advantage of permitting wide geographic studies across densely forested and inaccessible terrain. DNA sourced from scats also permits the collection of large sample sizes and, when compared to opportunistic sampling involving road kill or shelter animals, minimizes sampling bias.

Both microsatellite genotyping and sequencing of DNA isolated from koala scats have been shown to produce reliable results. Analysis of non-invasively isolated DNA using genomic methods, such as single nucleotide polymorphisms (SNPs), have been demonstrated (Fabbri et al., 2012; Snyder-Mackler et al., 2016) and may widen the applicability of DNA isolated from koala scats. Another potential benefit of DNA isolated from scats is that DNA from gut microbes or ingested plant material may also be present, which could be used to gather additional information, such as measures of health and dietary preferences (Bradley et al., 2007; Ley et al., 2008). Future advancements in technologies and methods will provide additional, and more efficient uses for non-invasive genetic sampling in conservation genetics. In the case of the koala, this has the potential to lead to standardized methods that can be analysed and compared across the koalas’ entire range providing a better understanding of population genetic diversity and promoting the conservation of this iconic species.

Data availability. Additional tables and figures referred to in the article text along with replicate and consensus genotype data used for this study are available in this article’s supplement.

The Supplement related to this article is available online at doi:10.5194/we-17-9-2017-supplement.

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Competing interests. The authors declare that they have no conflict of interest.

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Chapter 3 | Supporting information

DNA isolation method used for 2007 samples

Excerpt from Karsa, M.M. (2007) *Genetic analysis of koala populations using DNA extracted from faecal material*. Honours Thesis, Monash University, Churchill

Intestinal epithelial cells on the faecal pellets were collected by placing individual pellets in sterile screwtop vials (Cospak Pty. Ltd.) with 800 – 2000 µl phosphate- buffered saline (50 mM NaH₂PO₄.2H₂O, 50 mM Na₂HPO₄, 0.15 M NaCl, pH 7.4), sufficient to allow recovery of approximately 400 µl following the wash. The surface of the pellet was washed gently by rolling the vials on the Gyrotory Mixer (Ratek Instruments Pty. Ltd.) for eight minutes. 200 µl of the wash was removed and placed in a 2 ml-microcentrifuge tube. Another 200 µl of the wash was also removed and transferred into a second 2 ml-microcentrifuge tube.

DNA was extracted from the recovered epithelial cells using the QIAamp® DNA Stool Mini Kit (Qiagen Pty. Ltd.). Cells were lysed by the addition of 1.6 ml of Buffer ASL to each 200 µl extract, followed by incubation at 35°C for one hour. Samples were vortexed occasionally (once every 15-30 minutes) to ensure samples were thoroughly mixed. Samples were then centrifuged at 13000 x g (13200 rpm) for 1 minute to pellet the debris.

The supernatant (around 1.4 ml) was pipetted into a new 2 ml-microcentrifuge tube. One InhibitEX™ tablet was added to each sample to remove inhibitory materials that might be present. Samples were vortexed until the tablet dissolved, then incubated at room temperature for one minute to allow inhibitors to adsorb to the InhibitEX™ matrix. Samples were then centrifuged at 13000 x g (13200 rpm) for 25 minutes to pellet inhibitors bound to the InhibitEX™ matrix.

The supernatant (around 600 µl) was immediately removed into 2 ml-microcentrifuge tubes followed by addition of 20 µl of 20 mg/ml Proteinase K. 600 µl of Buffer AL was added to each sample; a homogenous solution was achieved by vortex mixing. The mixture was then incubated at 70°C for 10 minutes. DNA was precipitated by the addition of 600 µl 99.9% ethanol to the lysate.

Around 680 µl of the first lysate from each pellet was pipetted onto QIAamp® spin columns before centrifuging at 13000 x g (13200 rpm) for one minute. The filtrate was discarded. This step was repeated so that both extracts from each pellet passed through a single column.

500 µl of washing buffer, Buffer AW1 was added onto the QIAamp® spin column before centrifuging at 13000 x g (13200 rpm) for one minute. Collection tubes containing the filtrate were discarded. The QIAamp® spin columns were then placed into new collection tubes. 500 µl of washing buffer, Buffer AW2 was added onto the columns before centrifuging at 13000 x g (13200 rpm) for 4 minutes. Centrifugation time was increased to ensure smooth downstream applications as residual Buffer AW2 in the eluate may inhibit subsequent PCR (QIAamp® DNA Stool Mini Kit Handbook, 2001). Collection tubes containing the flow-through were discarded.

After placing the QIAamp® spin columns into fresh, 2-ml microcentrifuge tubes, 100 µl of Buffer AE was pipetted directly onto the QIAamp® membrane. Columns were incubated at room temperature for five minutes, and then centrifuged at 13200 rpm for one minute to elute DNA. The elution step was repeated with another 100 µl of Buffer AE to increase DNA yield. The extracted DNA was stored at 4°C.

Table S1 Number of loci positively amplified and scored, and the proportion of available loci that were identical, in samples genotyped in duplicate. Molly and Marlo were sampled in both 2007 and 2013. All other duplicates were sampled in 2013.

Koala name	Positive loci	Identical loci
Molly (2013)	12	100%
Molly (2007)	11	
Marlo (2013)	12	100%
Marlo (2007)	8	
Mitta	12	100%
Mitta	12	
Mitta	12	
Lisa	12	100%
Lisa	11	
Lara	12	100%
Lara	12	
Lennox	10	100%
Lennox	10	
Han	12	100%
Han	11	
Vivian	12	100%
Vivian	12	
Bernie	12	100%
Bernie	12	

Table S2 Summary of putative parent-offspring displaying incompatible genotypes. Potential parents identified, based on the absence of any mismatching loci.

Parent	Offspring	Mismatched loci	Potential parent/s
Marrguk ♀	Lorien ♀	3	None identified
Nellie ♀	Kevin ♂	4	Jupiter ♀
Banjo ♂	Lisa ♀	6	Ganymede ♂
Vivian ♂	Lennox ♀	4	Mantis ♂, Nautilus ♂
Merv ♂	Lara ♀	1	Banjo ♂

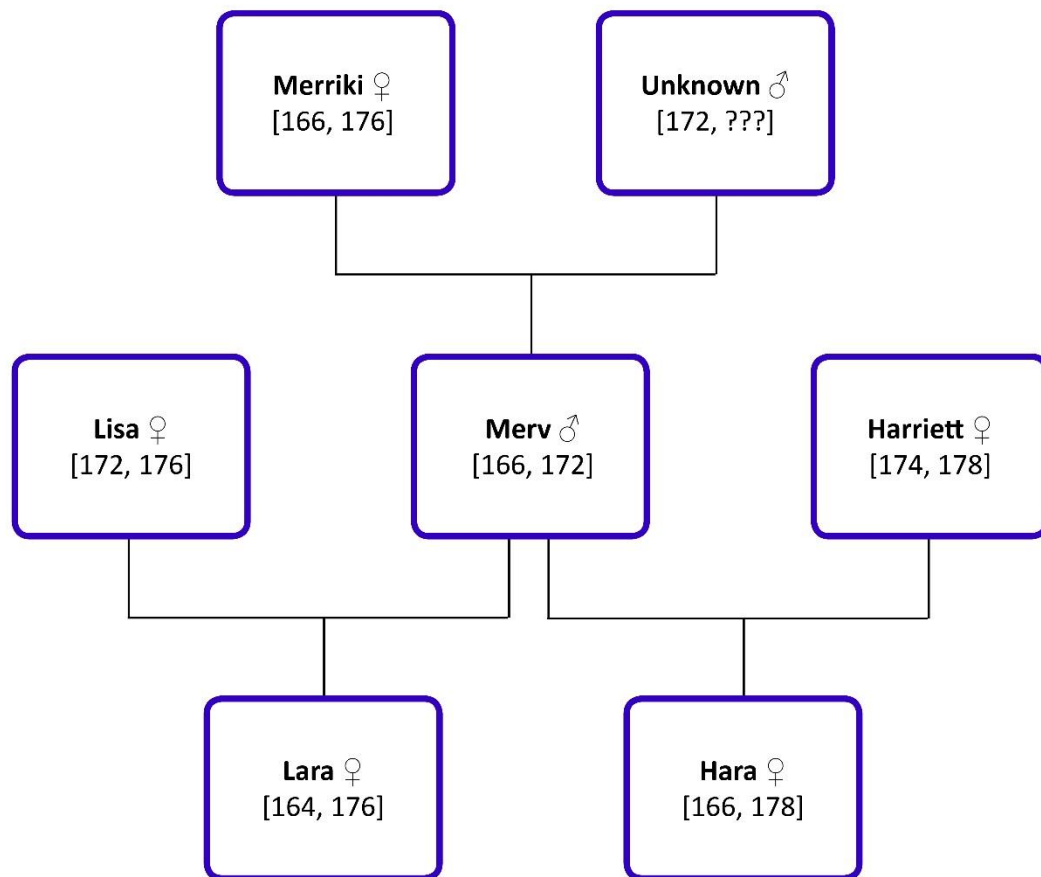


Figure S1 Pedigree for locus K2.1 where incompatible alleles were found between Merv and Lara. The 166 bp allele is not likely to be an error as it is present at K2.1 of his mother (Merriki) and another of his offspring (Hara). Two identical consensus genotypes were obtained independently for Lara. The 164 bp allele at K2.1 was present in an additional seven KCC individuals. The 164 bp allele is also not likely to be an error. Given Lisa as the known mother of Lara, CERVUS identified Banjo as the most likely father of Lara with a significant odds ratio obtained for the trio (Lara–Lisa–Banjo). Further analysis using additional markers may be required to confidently identify paternity for Lara.

Chapter 4 | foreword

Pedigree data in chapter three validated consensus genotypes obtained using the methods outlined in chapter two, thereby confirming that reliable genotypes are obtained. Chapter three also showed that the suite of twelve microsatellite markers used to genotype koalas was likely to provide unique genotypes, even where individuals were closely related, provided that data for more than ten loci were successfully obtained.

Estimations of the degree of relatedness between individuals is commonly used for ecological investigations using genetic data (e.g. estimating inbreeding, gene flow or animal movements). There are many different relatedness estimators to choose from, each of which may perform differently under particular situations. Choosing the most suitable estimator for a particular study system is therefore important to maximise the power of analyses using relatedness estimators (and conclusions based upon them). Chapter four evaluates the performance of seven different relatedness estimators and identifies the best performing estimator/s for use in our study system. This was done by calculating both genetic and pedigree based estimates of relatedness using both empirical (from individuals within the captive colony sampled in chapter three) and simulated data.

Another issue when using non-invasive sampling of DNA is that missing data, at some loci, is likely for a proportion of samples. Particular levels of missing data may result in inaccurate estimates of relatedness. Eliminating all samples with some missing data may, however, reduce analytical power. Chapter four, therefore, also assesses the effect of missing data on relatedness estimates between individuals in the pedigree, in order to gauge the level of missing data that may be acceptable when utilising analyses based on relatedness estimates.



Chapter 4

The performance of relatedness estimators for wildlife studies: an evaluation using empirical and simulated data





Chapter 4

The performance of relatedness estimators for wildlife studies: an evaluation using empirical and simulated data

Abstract

Molecular estimates of relatedness (r) are useful for a range of studies on wild animal populations including the inference of kinship structure, patterns of dispersal and barriers to gene flow. The results of such investigations often inform management decisions, so maximising the accuracy and power of these methods is important. Many relatedness estimators are available, the performance of which may vary depending on aspects of the population being investigated, such as relatedness composition, a factor which is commonly unknown. Despite potentially substantial differences in estimator performance, estimator choice is rarely based on comparisons of performance, but rather on the basis of those used in other studies.

In order to assess the performance of estimators, we used both empirical data (genotypes from DNA sourced non-invasively from a pedigreed koala population) and simulated data to compare seven relatedness estimators available in the R package, *related*, taking into account a population's relatedness structure and the presence of missing data, a common issue where non-invasive sources of DNA are used.

It was found that even when the relatedness structure of a population is unknown, a clear indication of the best estimator/s to use can be obtained. Using the best performing estimator, the proportion of pairwise comparisons with changes in r greater than 0.125 due to missing data ranged from 10% (one missing locus) to 31% (four missing loci). Evaluating the best

estimator to use for a particular study is a worthwhile and important step for any study utilising relatedness estimators.

Introduction

Inferring genetic relatedness between individuals is a common tool in the field of molecular ecology. Measures of relatedness (r) estimate the probability that alleles from two individuals are identical by descent (Jones & Wang 2010) and can be calculated between individuals or averaged across many pairs (e.g. spatial autocorrelation; Hardy & Vekemans 2002). Due to higher levels of variation in individual estimates, analyses using averaged relatedness values are generally more robust (Taylor 2015). Molecular estimates of relatedness can aid ecological investigations of kinship structure and inbreeding in natural populations, especially where other indicators of relatedness, such as parental interactions or mating events, are unclear or difficult to observe (e.g. Walker *et al.* 2008). Relatedness estimates can aid in the reconstruction of wild pedigrees (Stenglein *et al.* 2011) and are also used for landscape genetic studies, which can reveal patterns of gene flow and dispersal (Frantz *et al.* 2010; Lachish *et al.* 2011) and may inform our understanding of how populations have colonised or moved across a landscape. In two populations of Eurasian badger (*Meles meles*), for example, Frantz *et al.* (2010), estimated pairwise relatedness and identified greater dispersal distances in one population compared to the other, providing an indication that differing patterns of dispersal in Eurasian badger populations may be driven by differences in population density and habitat quality. Results from relatedness analyses can therefore provide important species information with the potential to inform conservation and management strategies.

A multitude of pairwise relatedness estimators are available, though their performance may vary depending on the attributes of the chosen estimator (Wang 2011) and the population under investigation (Csilléry *et al.* 2006). Estimator performance may be affected by the number of and levels of polymorphism in the markers used and the proportion of related and unrelated individuals present in the sampled population (Van de Casteele *et al.* 2001; Csilléry *et al.* 2006; Pew *et al.* 2015). To compare the performance of estimators for a population,

genotypes of various pairs of individuals (e.g. parent-offspring, full-sibling, half sibling and unrelated) can be simulated and used to evaluate estimator accuracy for a particular dataset (e.g. Van de Casteele *et al.* 2001). Simulations also provide information regarding the expected distribution of relatedness estimates according to relationship, thereby providing the ability to calculate confidence intervals and assess the dependability of particular estimates (Konovalov *et al.* 2004; Wang 2011).

Investigations of best estimator choice are often not undertaken (Taylor 2015) despite potentially substantial differences in estimator performance and the availability of programs, such as *related* (Pew *et al.* 2015) and COANCESTRY (Wang 2011), which provide user friendly means by which the best estimator for a particular study may be assessed. Given that the results of a study using relatedness estimators may be used to guide conservation strategies, it is important to ensure that the most accurate estimator for the dataset in question is used. The large number of available relatedness estimators can, however, make choosing the most appropriate estimator for a particular study or research question difficult.

Additionally, prior knowledge of the number of related individuals in the sampled population is often unknown and may also make ascertaining the best estimator for a particular study challenging (Csilléry *et al.* 2006). Choosing a relatedness estimator based on factors other than comparisons of performance in the study system could potentially weaken the results that are obtained. A more appropriate estimator could provide more accurate results, or highlight differences that may not be otherwise be evident.

Options for obtaining genetic samples from wildlife include both invasive and non-invasive methods. Invasive sampling involves animal capture and collection of blood or tissue biopsies directly from the animal while non-invasive genetic sampling involves the isolation of DNA from shed biological materials such as scats (e.g. Stenglein *et al.* 2011), hairs (e.g. Walker *et al.* 2008) or feathers (e.g. Hogan & Cooke 2010). Invasive sampling can be costly, time

consuming and arduous, thereby placing limitations on the number of individuals that can be sampled. Another advantage of using non-invasive sampling for relatedness studies is that sampling all individuals in a population may be possible (e.g. Walker *et al.* 2008) which can increase reliability when inferring relatedness where prior relationship information is not available (Jones & Ardren 2003).

A major challenge with non-invasive DNA sampling is that levels of DNA quantity and/or quality may be reduced, which can result in increased error rates and amplification failure (Pompanon *et al.* 2005). The use of non-invasively sourced DNA therefore requires thorough method optimisation and assessment of rates of PCR success and genotyping error to maximise data quality (Pompanon *et al.* 2005; Beja-Pereira *et al.* 2009). Obtaining good quality DNA is largely dependent on obtaining good quality samples in the first instance which can be achieved in the field by attempting to collect the freshest samples possible that have not been exposed to rain (e.g. Wedrowicz *et al.* 2013) and utilising appropriate sample collection and storage methods for the sample type in question. Biological molecules that may interfere with DNA analyses utilising PCR (PCR inhibitors) are often co-isolated with DNA extracted from non-invasive sources. PCR inhibitors and/or low amounts of target DNA can result in repeated instances of amplification failure, leading to missing data within final multi-locus genotypes. Discarding genotypes with missing data may be important where a particular level of certainty is required, however, in other instances, removing genotypes may weaken a study as a large amount of useful data might also be discarded unnecessarily. Another consideration is that some samples may provide greater value than others, such as individuals sampled in areas where the species is at extremely low density or where few samples were collected. Knowledge of how missing data impacts relatedness estimates in a particular system would be useful in order to retain as many samples as possible and to maximise the

data obtained from potentially valuable samples, thereby minimising the loss of time and money associated with discarded data.

The availability of data from a population with documented pedigree information provides an opportunity to empirically assess the ability to infer relatedness for a particular population and suite of markers. This study considers genotypic data obtained using DNA collected non-invasively from a captive, pedigreed koala population, in order to determine the most accurate relatedness estimator/s for analyses of southern koala populations. The relatedness composition of a population and, missing data (a common feature of datasets using DNA obtained from non-invasive sources) may both impact the performance of relatedness estimators. The specific objectives of this study are, therefore, to compare the performance (using regression analysis and rates of misclassification) of seven relatedness estimators based on both empirical (pedigree data confirmed by genetic data) and simulated data, taking into consideration relatedness structure (different proportions of related pairs within a population) and the effect that missing data may have on relatedness estimates. The outcomes of this study will guide the best choice of relatedness estimator/s for southern koala populations and may also serve to demonstrate the assessment of relatedness estimator performance in other study systems where the proportion of related pairs within a population is unknown.

Methods

Pedigree microsatellite genotype dataset

Microsatellite genotypes were obtained from DNA isolated from scats collected at the Koala Conservation Centre (KCC) located on Phillip Island, Victoria, Australia (for details see Wedrowicz *et al.* 2017). Consensus DNA profiles were obtained for 32 koalas at the KCC. Rates of allelic dropout and false alleles, calculated from replicate genotypes, were 1.9% and 0.2% respectively. Tests for null alleles (Van Oosterhout *et al.* 2004) were negative. Parent-

offspring relationships in the pedigree were confirmed by checking that consensus microsatellite genotypes followed Mendelian rules of inheritance. Five incompatible relationships were identified and the relationship subsequently classified as unknown. In total, the genetic data consisted of 21 individuals with complete twelve marker genotypes; three individuals with missing data at one locus; four individuals with missing data at two loci; one with three missing loci; two with four missing loci and one with seven missing loci. By examining the distribution of the number of mismatched loci between individual genotypes (Paetkau 2003) and estimating the probability of identity (Waits *et al.* 2001), it was found that individuals could be reliably discriminated where data were missing at two loci or less (P_{ID} and $P_{ID_{sibs}}$ for the ten least informative loci was 2.5×10^{-6} and 2.8×10^{-3}). The four individuals with missing data at more than two loci were therefore excluded from further analyses and a total of 28 individuals from the captive pedigreed population used for analysis. All 28 individuals had unique genotypes. The highest number of matching loci identified was nine (out of twelve), found between a pair of individuals who were full siblings. The consensus genotype data for the 28 individuals from the pedigreed koala population are referred to as the empirical dataset throughout.

Calculation of pedigree based relatedness

Relationships between individuals in the KCC population were obtained from KCC records and relationships confirmed using the genetic data, as outlined above (data not shown). Pedigree relationship coefficients (R) for each pair of individuals were calculated for the 28 individuals using the pedigree data (confirmed by the genetic data) and the *pedantics* package (Morrissey & Wilson 2010) in the program R (R Core Team 2014). Software for carrying out simulations usually consider parent-offspring ($R=0.5$), full sibling ($R=0.5$), half sibling ($R=0.25$) and unrelated ($R=0$) pairs and do not include more complex relationships (e.g. maternal half siblings, where the father of one sibling is the grandfather of the other,

$R=0.375$). The *pedantics* package calculates pedigree coefficients based on all pedigree relationships, thereby accounting for all relationship types that may produce a particular R value (e.g. $R=0.25$ would be represented by any relationship, or complex combination of relationships, existing between two individuals that would produce an R value of 0.25, for example, half-siblings, double first cousins and avuncular relationships all have an R value of 0.25). Parentage information was omitted where the parents of an individual koala were unknown or uncertain (due to discrepancies between the pedigree and genetic data and an inability to infer alternative parental relationships), meaning that some pairwise relationships may have been classified as unrelated when the true degree of relationship may have been higher. Parent-offspring are expected to share a common allele at every locus. Allele sharing by descent is therefore expected to be exactly 0.5 at autosomal loci while other relationship categories are expectations that are variable. Values calculated by the *pedantics* package (derived from the pedigree data) were used to assign each pairwise relationship as: first order (I: parent-offspring (PO) or full siblings (FS), $R \geq 0.50$), second order (II: half siblings (HS), avuncular or grandparent-grand offspring, $0.25 \leq R < 0.50$) or unrelated (UR: $R < 0.25$). A summary of the datasets and methods used in this study are shown in Fig. 1 and are described in the following methods sections.

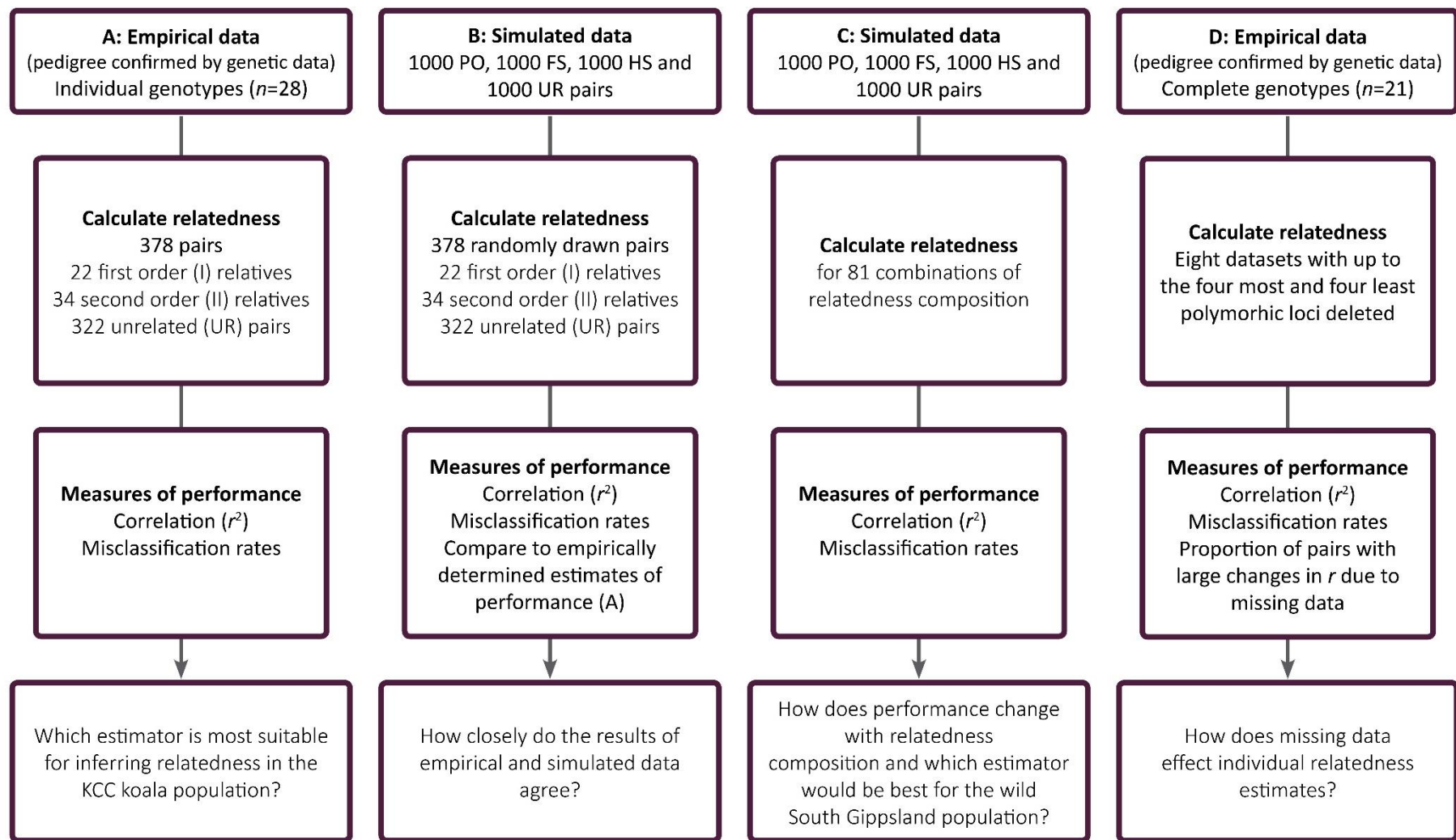


Figure 1 Summary of the datasets and analyses used in this chapter

Microsatellite marker based relatedness

Estimates of pairwise relatedness were calculated between all individuals in the KCC population using the seven estimators available in the R package *related* (Fig. 1A; Pew *et al.* 2015). These were the triadic likelihood estimator (TML; Wang 2007), Wang's (2002) estimator (WNG), the Li *et al.* (1993) estimator (LYL), Lynch and Ritland's (1999) estimator (LYR), Ritland's (1996) estimator (RIT), Queller and Goodnight's (1989) estimator (QGN) and the dyadic likelihood estimator (DML; Milligan 2003). Estimated values of genetic relatedness (r : *related* output) were then compared to known relatedness (R: *pedantics* output). Two methods were used to compare performance between estimators; calculation of the variance (r^2) in linear models explaining known relationships as per Van de Castelee *et al.* (2001) and estimation of relationship misclassification rates as described by Blouin *et al.* (1996).

Simulated data

To compare simulation data with the set of empirical data used here, 1000 twelve marker genotype pairs were produced in *related* (for each of PO, FS, HS and UR; 4000 individuals in total), using the allele frequencies for the pedigreed koala population (Fig. 1B). A second simulated dataset using allele frequencies obtained from a large, presumably outbred, population of koalas from the South Gippsland, Victoria (SG, $n=67$) was also produced to evaluate estimator performance in this wild koala population.

Calculation of r^2

Linear models were produced between pedigree relatedness and genetic relatedness for each estimator and the amount of variance explained (r^2) calculated. For the simulated dataset, the same number of related pairs (determined by the pedigree data) as the empirically sampled

koala population were randomly chosen from the pairwise relatedness estimates; 22 first order (I) relatives (randomly sampled from PO pairs and FS pairs), 34 second order (II) relatives and 322 unrelated (UR) pairs. Sampling was repeated 100 times, r^2 calculated for each repetition and the mean and standard error for the mean calculated.

Calculation of misclassification rates

Misclassification rates were used to compare the performance of estimators (i.e. not to discriminate relatedness groups) as relatedness estimates represent a continuum rather than discrete categories (Csilléry *et al.* 2006). Confidence intervals for the mean r value of each relationship category were calculated by bootstrapping. The midpoints between means of each relatedness category were designated as cut off points in order to classify relatedness estimates (as per Blouin *et al.* 1996) according to categories I, II, or UR. The relatedness category genetically assigned to each pair of individuals was then compared to the pedigree assigned groups. For the simulated dataset, random sampling of 22 (6%) first order (I) relatives (randomly sampled from PO pairs and FS pairs), 34 (9%) second order (II) relatives and 322 (85%) unrelated (UR) pairs determined by the pedigree data and classification into categories based on the genetic data were repeated 100 times and the mean and standard error of the mean reported.

The effect of relatedness composition on estimator performance

To investigate the effect of differing population relatedness structure, pairwise relatedness estimates were sampled (from the 4000 simulated) and r^2 calculated for various hypothetical population compositions made up of all combinations of 0.1, 1, 5, 10, 15, 20, 25, 30 and 35% first or second degree relatives (resulting in a total of 81 different population compositions; Fig. 1C), with the remaining proportion of the population classified as unrelated. Sampling and calculation of r^2 was performed 100 times and averaged for each population composition.

The effect of missing data

Complete consensus genotypes were used ($n=21$) to investigate the impact of missing data, resulting in 210 pairwise comparisons made up of 12 (6%) first order relative (I), 17 (8%) second order relative (II) and 181 (86%) unrelated (UR) pairs (Fig. 1D). From the dataset of 21 individuals, data were sequentially deleted from the four most and four least informative loci, representing worst and best case scenarios given our limit of missing data at a maximum of two loci for each individual. For example, the best case scenario would be missing data at one of the less informative polymorphic loci between a pair of individuals for which relatedness would be calculated and the worst case scenario where a total of four highly polymorphic loci (two different loci in each individual) between a pair of individuals was missing.

Specifically, data were deleted at the four most polymorphic loci: K2.1 (11 loci), both K2.1 and Pcv6.3 (10 loci), K2.1, Pcv6.3 and Pcv2 (9 loci) and all of K2.1, Pcv6.3, Pcv2 and K10.1 (8 loci), and from the four least polymorphic markers where data were deleted at Phc13 (11 loci), both Phc13 and Pcv31 (10 loci), Phc13, Pcv31 and Pcv24.2 (9 loci) and all of Phc13, Pcv31, Pcv24.2 and Pcv25.2 (8 loci). Misclassification rates and r^2 were calculated for each of the eight sets of data. Increases or decreases of over 0.125 in individual pairwise relatedness estimates were also summarised and compared.

Results

Comparison of relatedness estimators

Compared to known pedigree relationships for the captive koala population, the triadic (TML) and dyadic (DML) maximum likelihood estimators accounted for the greatest amount of variation in the data with r^2 values of 35.1% and 34.5% and were followed by the LYR estimator with an r^2 value of 32.6% (Table 1). Scatterplots with linear regressions are shown in Fig. S1 of the supporting information. When considering rates of correct relationship assignment, TML and DML also performed best with 77.5% and 75.7%, respectively, of pairwise relationships being correctly assigned overall (Table 1). The LYR estimator had the next highest proportion of overall correct classifications with 73.5% of pairwise relationship assigned to their correct category. The remaining five estimators produced overall correct identifications between 64.0% and 69.6%.

Estimator performance varied depending on the relationship being assigned (Table 1). Relatedness estimates using the WNG estimator correctly assigned 91% of known first order relatives, while the TML and DML estimators performed best for the classification of unrelated pairs to their respective categories (Table 1: 82% and 80%, respectively) while also assigning more than 80% of first order relatives to their correct category. Conversely, the proportion of correctly assigned second order relatives was lowest for TML and DML (TML: $29 \pm 8\%$; DML: $26 \pm 8\%$) as well as LYR ($29 \pm 8\%$), while correctly assigned second order relatives were higher for all other estimators (Table 1: LYL: $50 \pm 9\%$; WNG: $44 \pm 9\%$; RIT: $44 \pm 9\%$; QGN: $41 \pm 9\%$). The types of misclassification (e.g. 'I' misclassified as 'II' or 'UR') made occurred at similar rates for all estimators except for unrelated individuals misclassified as second degree relatives which occurred around half as much using the DML and TML estimators ($\approx 10\%$; Table S1) compared to all other estimators ($\approx 20\%$).

Empirical and simulated data comparison

Based on the proportion of correctly identified pairwise relationships and r^2 values (Table 1, Table S1), the TML estimator was selected as the most appropriate for estimating relatedness in this particular koala population, though the DML and LYR estimators were also found to have similar levels of performance. Average TML relatedness scores for the empirical data corresponded well to relationship categories (Fig. 2; Fig. S2) and were in agreement with theoretical values (0.5 for first degree relatives and 0.25 for second degree relatives). Mean TML relatedness values differed significantly ($p < 0.005$) between first order relationships ($r = 0.50 \pm 0.08$), second order relationships ($r = 0.26 \pm 0.07$) and unrelated individuals ($r = 0.08 \pm 0.01$).

In general, patterns of r^2 and correct classification rates were comparable for the empirical and simulated data. Density plots displayed similar profiles between the empirical and simulated data for all estimators assessed (Fig. 3; Fig. S3). The three best performing estimators (based on r^2 and rate of correct classifications) were TML, DML and LYL for both the empirical and simulated datasets (Table 1). The different measures of estimator performance (rates of correct classification and r^2) ranked estimators identically for the empirical data and near identically using simulated data, where the order of some similar performing estimators was reversed (e.g. WNG and LYL were ranked 6th and 7th by r^2 and 7th and 6th using correct classifications; Table 1).

Table 1 Variation explained (r^2) and percent of pairwise relationships correctly assigned for the empirical and simulated datasets. Results for the empirical data are shown on the top line of each row, while results for the simulated data are shown on the second line of each row in italics and parentheses. Bold values indicate the best performing estimator in each category for both the empirical and simulated data.

		TML (%)	WNG (%)	LYL (%)	LYR (%)	RIT (%)	QGN (%)	DML (%)
Variance explained (r^2)		35.1	28.1	22.7	32.6	25.1	19.5	34.5
		(38.9 ± 0.21)	(26.8 ± 0.15)	(26.6 ± 0.15)	(37.0 ± 0.20)	(30.3 ± 0.23)	(28.5 ± 0.16)	(36.5 ± 0.19)
Percent correct classifications	I	81.8 ± 8.4	90.9 ± 6.3	72.7 ± 9.7	81.8 ± 8.4	63.6 ± 10	81.8 ± 8.4	86.4 ± 7.5
		(81.5 ± 0.83)	(80.4 ± 0.85)	(72.4 ± 0.95)	(78.1 ± 0.88)	(56.5 ± 1.1)	(78.5 ± 0.88)	(79.8 ± 0.86)
	II	29.4 ± 7.9	44.1 ± 8.6	50.0 ± 8.7	29.4 ± 7.9	44.1 ± 8.6	41.2 ± 8.6	26.5 ± 7.7
		(33.8 ± 0.81)	(46.8 ± 0.86)	(44.0 ± 0.85)	(47.9 ± 0.86)	(40.1 ± 0.84)	(43.9 ± 0.85)	(37.6 ± 0.83)
	UR	82.3 ± 2.1	70.8 ± 2.5	76.1 ± 2.4	67.1 ± 2.6	70.8 ± 2.5	65.2 ± 2.7	80.1 ± 2.2
		(81.0 ± 0.22)	(71.2 ± 0.25)	(78.4 ± 0.23)	(71.3 ± 0.25)	(78.9 ± 0.23)	(73.4 ± 0.25)	(78.7 ± 0.23)
	Total	77.5 ± 2.2	69.6 ± 2.4	64.6 ± 2.5	73.5 ± 2.3	68.0 ± 2.4	64.0 ± 2.5	75.7 ± 2.2
		(76.8 ± 0.22)	(69.5 ± 0.24)	(69.6 ± 0.24)	(74.9 ± 0.22)	(74.1 ± 0.23)	(71.0 ± 0.23)	(75.1 ± 0.22)

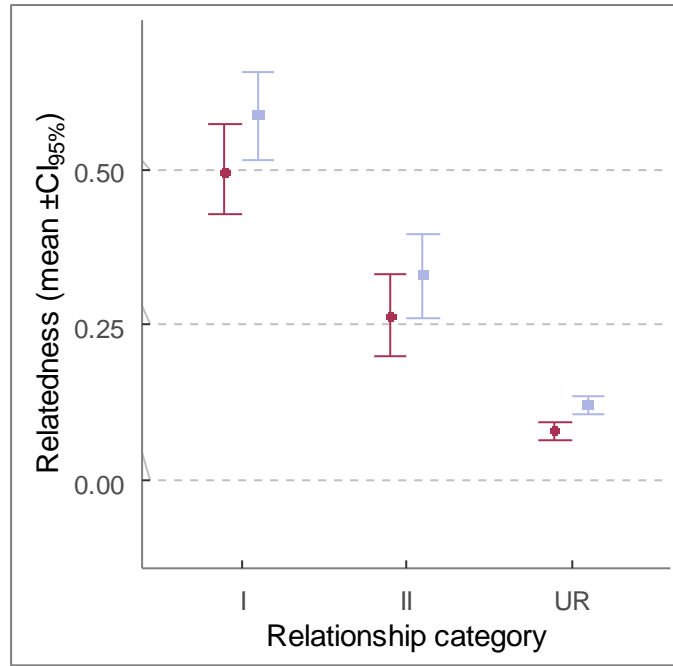


Figure 2 Differences in relatedness values according to known relationships within the empirical dataset (circles, dark shading) alongside results for the simulated dataset (squares, light shading). The simulated data consists of the same number of related individuals as the empirical dataset (I: 22, II: 34, UR: 322) drawn from 1000 simulated genotypes for each relationship category, 100 times. The graph shows 95% confidence intervals for the means between each relationship category which all differed significantly ($p < 0.0005$) from one another. **I**: First order relationships ($R \geq 0.50$); **II**: Second order ($0.25 \leq R < 0.50$) and **UR**: Unrelated individuals ($R < 0.25$). Comparisons for all estimators are shown in Fig. S2 of the supporting information.

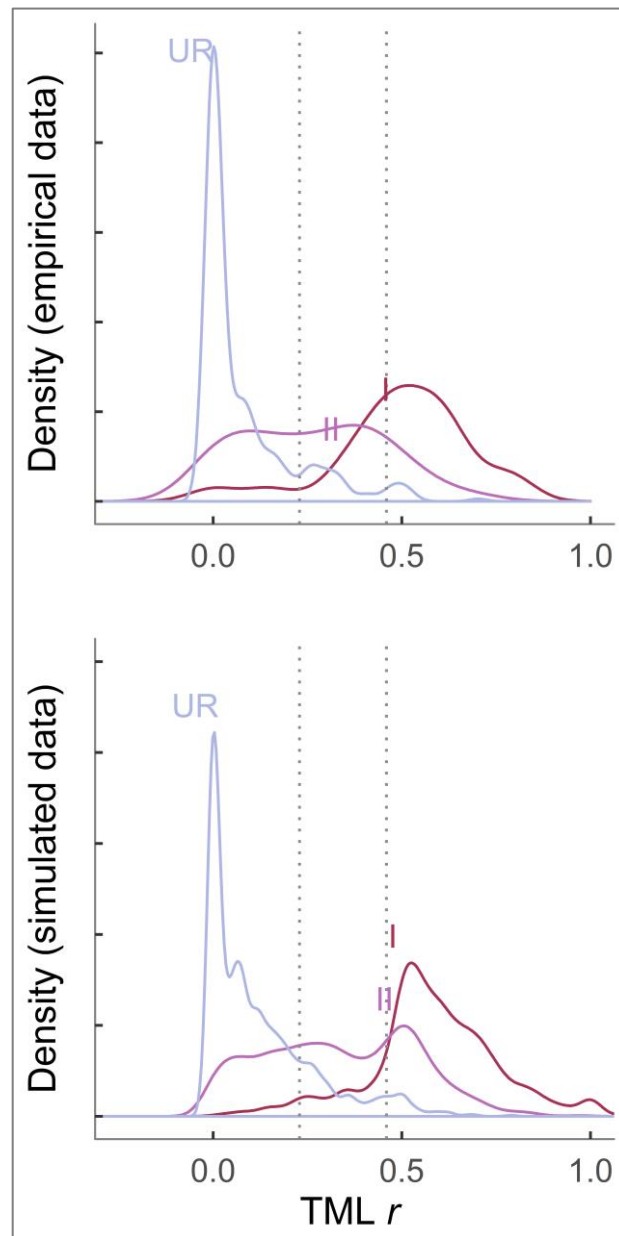


Figure 3 Density plots showing relatedness scores for first degree (I) relatives, second degree (II) relatives and unrelated (UR) pairs of individuals for the TML estimator. The simulated data consists of equal numbers of pairwise relationship (I: 22, II: 34, UR: 322) drawn 100 times from the simulated data (1000 genotypes for each category). Vertical dashed lines indicate the midpoints between means used for classification of individuals into relationship categories. Density data were similar for both empirical and simulated data across all estimators. Comparisons for all estimators are shown in Fig. S3 of the supporting information

Population relatedness composition

All estimators followed a similar pattern of change due to varying levels of population relatedness composition, with increasing proportions of second order relatives increasing r^2 below a particular proportion of first degree relatives and decreasing r^2 above that point (Fig. S4). The number of occasions that relatedness estimators outperformed others (on the basis of r^2) showed that TML performed best across all population compositions tested, followed by DML, LYR, QGN, WNG, LYL and RIT (Fig. 4). Results were similar using allele frequencies from the wild South Gippsland koala population (Fig. S5), indicating that the best performing estimators are likely to be the same for that population. Across the 81 population proportions tested, TML performed better (had a higher r^2) than the LYR estimator 54.3% of the time and better than the other five estimators more than 90% of the time. The difference between the TML and LYR estimators was small with the LYR estimator having consistently higher r^2 values (of 4% or less) when the proportion of second degree relatives was 25% or over and when the proportion of first degree relatives was very low compared to the proportion of second order relatives (e.g. 0.1 and 15%, 1 and 15%, etc.). The DML estimator also compared similarly to TML with differences of 3% or less in values of r^2 . Compared to TML the QGN, LYL, WNG and RIT estimators had mean differences in r^2 of up to 14, 16, 16 or 19%, respectively.

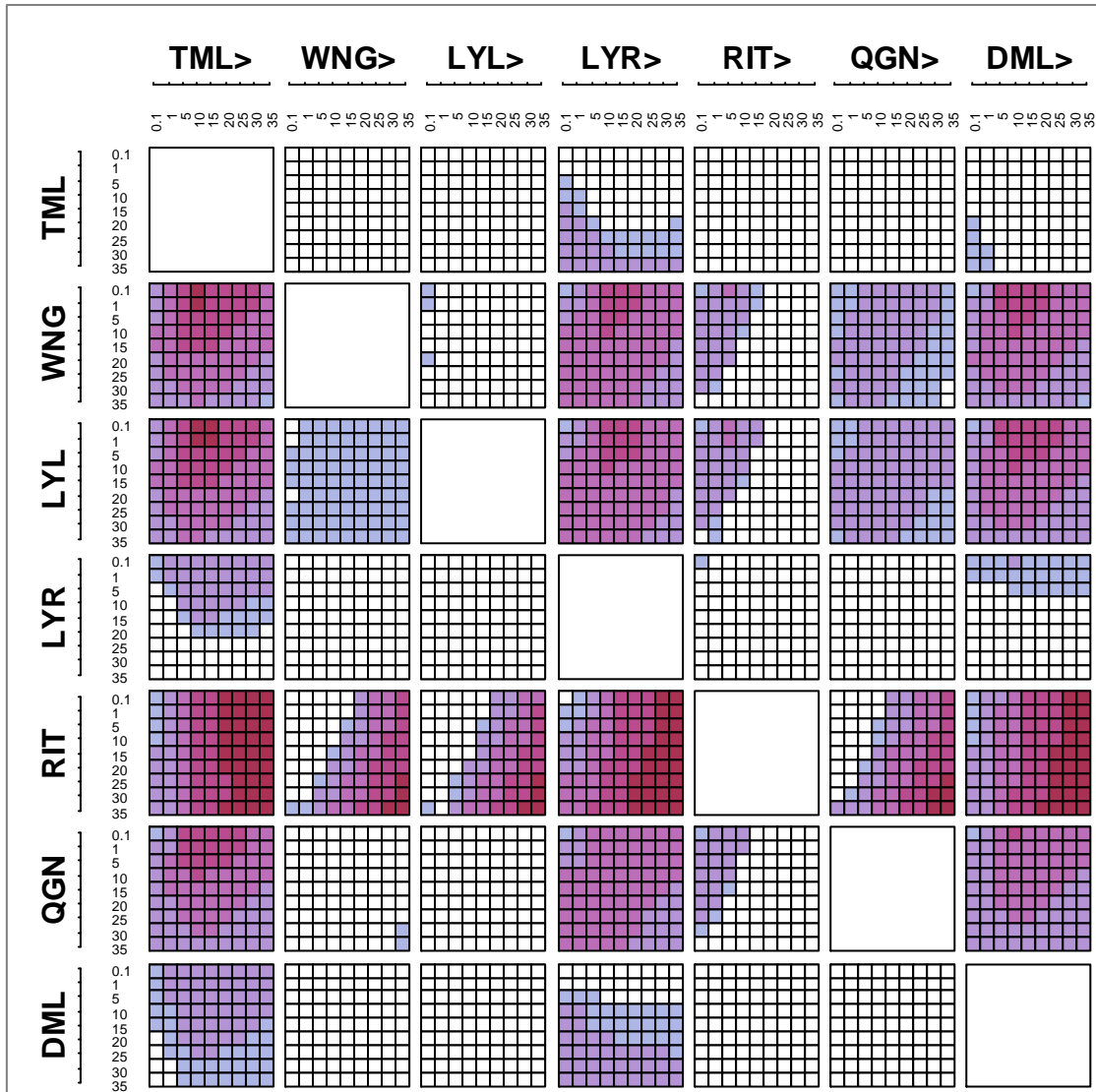


Figure 4 Comparison of r^2 between all estimators tested across 81 different relatedness compositions. The performance of estimators in columns are compared to those in rows. Comparison of each pair of estimators is made up of a 9 x 9 matrix representing the 81 population compositions tested. Columns (from left to right) of each matrix represent the proportion of first degree relatives (0.1, 1, 5, 10, 15, 20, 25, 30 and 35%) while rows (from top to bottom) indicate the proportion of second degree relatives (0.1, 1, 5, 10, 15, 20, 25, 30 and 35%) in the population. Shaded matrix cells indicate that the corresponding column estimator had a higher r^2 than the corresponding row estimator at the particular population composition. Unshaded cells indicate that the column estimator had a lower r^2 value than the row estimator. Different levels of shading indicate the magnitude of the increase in r^2 at five levels: <1% (lightest shading), 1-5%, 5-10%, 10-15% and >15% (darkest shading). All five shades are present in the 9 x 9 matrix at row 5, column 4 (LYR>RIT). The same figure using allele frequencies from the South Gippsland koala population are shown in Fig. S5.

The effect of missing data

Values of r^2 associated with each estimator became more similar where data were missing at the four most polymorphic loci ranging from 8% to 14% (Table S3). Missing data at the four most informative loci reduced r^2 from approximately 40% to 11% for the TML, LYR and DML estimators (Table S3). In contrast, missing data at the four least informative loci reduced r^2 from around 40% to 32% (Table S2). The overall percentage of pairwise comparisons correctly identified by the TML estimator dropped from $77.6 \pm 2.9\%$ using the entire 12 loci to $66.7 \pm 3.3\%$ when data were missing from genotypes at the four most informative loci (Table S5).

Changes in r^2 and correctly classified relationships due to missing data presented here are representative of data missing between all pairs of individuals. Large changes (>0.125) in individual pairwise estimates were therefore also examined. The proportion of pairs of individuals with large changes in relatedness (over 0.125) due to missing data at four of the most informative loci differed between estimators. Missing data at K2.1, Pcv6.3, Pcv2 and K10.1 (two loci missing for each individual) resulted in changes in relatedness estimates of over 0.125 for 20% of pairs using the RIT estimator and 68% of pairs using the WNG estimator (Fig. 5). The percentage of pairwise relatedness estimates changing by more than 0.125 due to missing data at the four most informative loci were 31%, 37% and 44% for the TML, DML and LYR estimators respectively (Fig. 5). Increases in genetic estimates of relatedness were generally more common than decreased estimates (Fig. 5). Where the four most polymorphic loci were lost, TML relatedness estimates for 13 pairwise relationships out of 210 (6%) decreased by more than 0.125 (9/13 related and 4/13 unrelated), while an additional 53 pairwise relationships out of 210 (25%) increased by more than 0.125 (8/53 related, 45/53 unrelated).

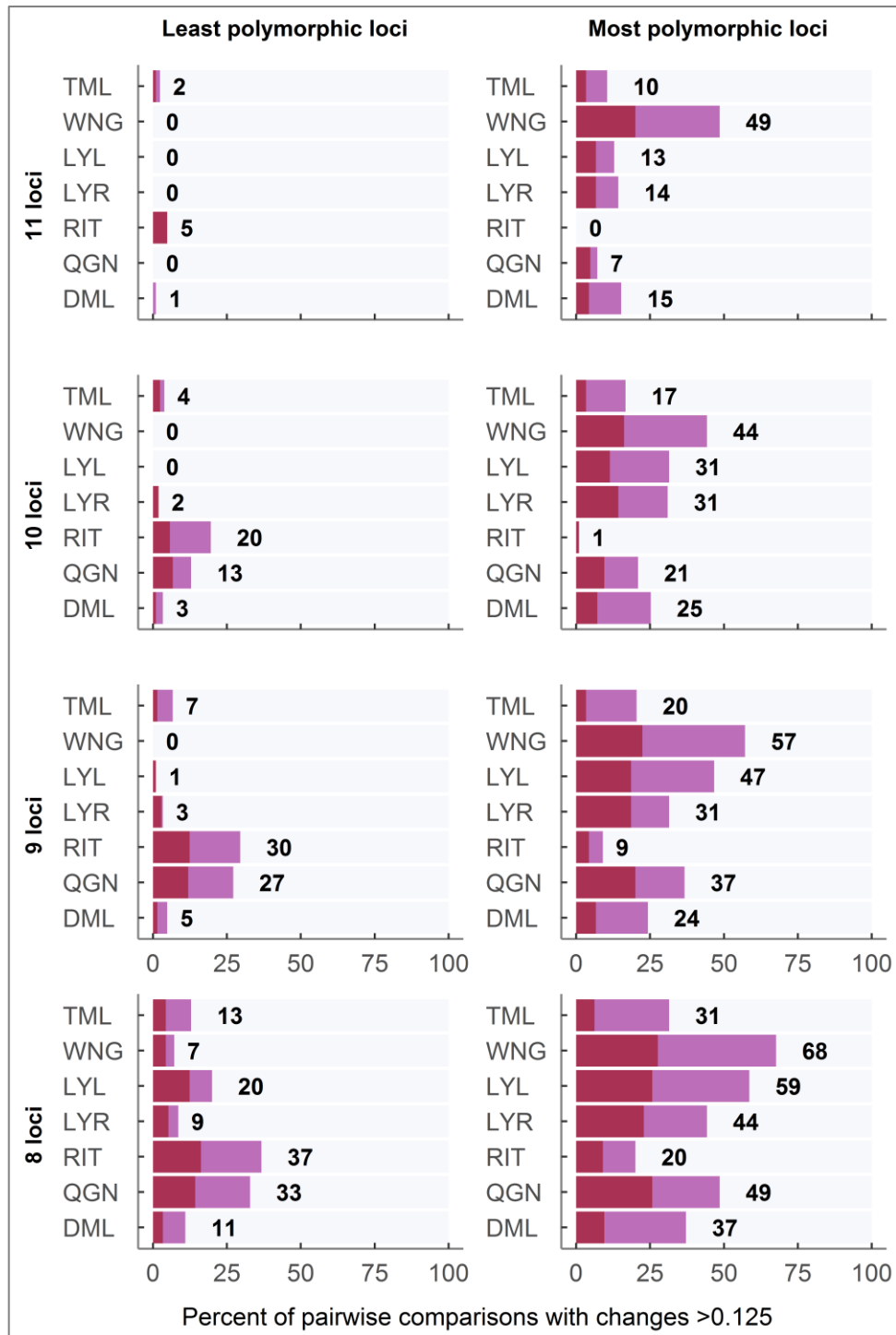


Figure 5 The effect of missing data on relatedness estimates for the empirical data. Bar graphs show the percentage of pairwise relationships with either a decrease (darkest shading) or increase (intermediate shading) of more than 0.125 in relatedness estimate. Graphs on the left hand side show changes due to sequential loss of the four least polymorphic markers (Phc13, Pcv31, Pcv24.2 and Pcv25.2) for the empirical dataset. Graphs on the right hand side show changes due to sequential loss of the four most polymorphic markers (K2.1, Pcv6.3, Pcv2 and K10.1) for the empirical dataset.

Discussion

We have used genotypic data from a captive breeding population with pedigree data to evaluate the most appropriate relatedness estimator for two Victorian koala populations, the results of which also highlight the importance of choosing a relatedness estimator based on evaluation of performance for a particular sample set. The relatedness estimators considered in this study explained between 19.5% and 35.1% of known pedigree relatedness for the KCC koala population. We found that the TML estimator was the most accurate for estimating relatedness in all datasets analysed and was closely followed by the DML and LYR estimators. Both measures of performance (r^2 and misclassification rates) provided near identical rankings of the five top performing estimators indicating that either one of these measures should be sufficient to assess differences between estimators. Differences in the discrimination of particular relatives were also evident. Choice of relatedness estimator may, therefore, also depend on the research question. For example, if the objective of a study was focussed on discrimination of first order relatives only, in this case, the WNG or DML estimators may be most appropriate, while if greater discrimination of second order relatives was required, an estimator other than the maximum likelihood estimators might be preferable. Though particular estimators stood out in the dataset used here, this may not be true for other datasets where other estimators may perform better because of differences in the number of loci used, allele frequencies, the structure of related individuals in the population and the question being asked.

It was shown that even when the relatedness composition of a population is unknown, it is possible to gain insight into the most appropriate estimator/s to use. In this system, all estimators showed a general decrease in variability (r^2) with increasing proportions of first degree relatives (Fig. S4). For example, r^2 for TML estimates of relatedness was $1.8 \pm 0.11\%$ when the proportion of first and second degree relatives was 0.1% and 0.1% respectively; 56

$\pm 0.17\%$ when the proportion of first and second degree relatives was 20% and 20% and $66 \pm 0.13\%$ when the proportion of first and second degree relatives was 35% and 0.1%. This result is similar to that obtained by Csilléry *et al.* (2006) who suggest that the composition of relatedness within a population is the major driver of estimator performance and may determine the maximum level of variance that is able to be explained by relatedness estimators.

The best performing estimator could change depending on relatedness composition. Where the proportion of second degree relatives was low (below about 25%) the TML estimator was best, while when it was high (over 25%) the LYR estimator performed better, although the difference between the two was small ($\approx 4\%$). Greater differences in estimator performance (than found here) could be possible in other study systems so evaluation of relatedness estimators across differing proportions of related pairs is therefore worthwhile. Where information about relatedness composition is unknown, and more than one estimator is found to perform well over the range of relatedness proportions evaluated, it may be suitable to use both estimators for analysis and choose the most biologically relevant results where differences, if any, are evident.

Samples with missing data might ideally be excluded from analyses, however, relatedness estimates might only be significantly altered for a small number of individual pairs in the dataset, depending on the number and polymorphic loci that are missing. Missing data effected relatedness estimates of some pairs of individuals more greatly than others. The TML estimator was found to perform well for this dataset and it also tended to produce the fewest large changes in relatedness estimates (after RIT). Where data were missing at the four least polymorphic loci, large changes in TML estimates of relatedness were found to occur in 27 of the 210 (13%) pairwise relationships, while data missing at the four most polymorphic loci resulted in large changes in 66 of the 210 (31%) pairwise relationships. This result may be

used as a guide, suggesting that a pair of individuals whose genotypes have missing data at any four loci would have a 13–31% chance of a change in relatedness estimate exceeding 0.125. This may be decreased to 7–20% by limiting the number of missing loci between a pair of individuals to three, or to 4–17% by limiting the number of pairwise loci that are missing to two. It may be useful to identify the number of loci missing, along with a rank of their combined variability, to highlight pairs whose relatedness estimate may be less reliable than others. The stringency with which researchers decide to impose limits for missing data may depend on whether individual pairwise estimates (e.g. identification of close relatives in a population) or averaged pairwise estimates (e.g. spatial autocorrelation) are being used, since large changes in relatedness estimates are more likely to affect results making comparisons between individuals.

Differences in performance can vary between the discrimination of particular relatives and an estimator's overall ability to infer relatedness. The main factor influencing the accuracy of genetic relatedness estimates appears to be the number of related individuals in the population. The proportion of related individuals that are sampled is however, unlikely to be known, but by, evaluating estimator performance over a wide range of relatedness structures a clear indication of the most appropriate estimator/s to use can be obtained. As recommended previously (Van de Casteele *et al.* 2001; Wang 2011), the performance of relatedness estimators should be compared on a case by case basis using simulations, though this step is often neglected (Taylor 2015). It has also been pointed out by Taylor (2015) that studies using relatedness estimators can be difficult to compare due to differences in estimator performance and no standard method by which to report performance. The development and expansion of computer software and packages available in R to simplify these analyses as well as a potential future increase in the number of studies reporting estimator performance for their

dataset is likely to help facilitate such investigations, a crucial step if subsequent analyses involving relatedness estimates are to be as accurate and reliable as possible.

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Chapter 4 | Supporting information

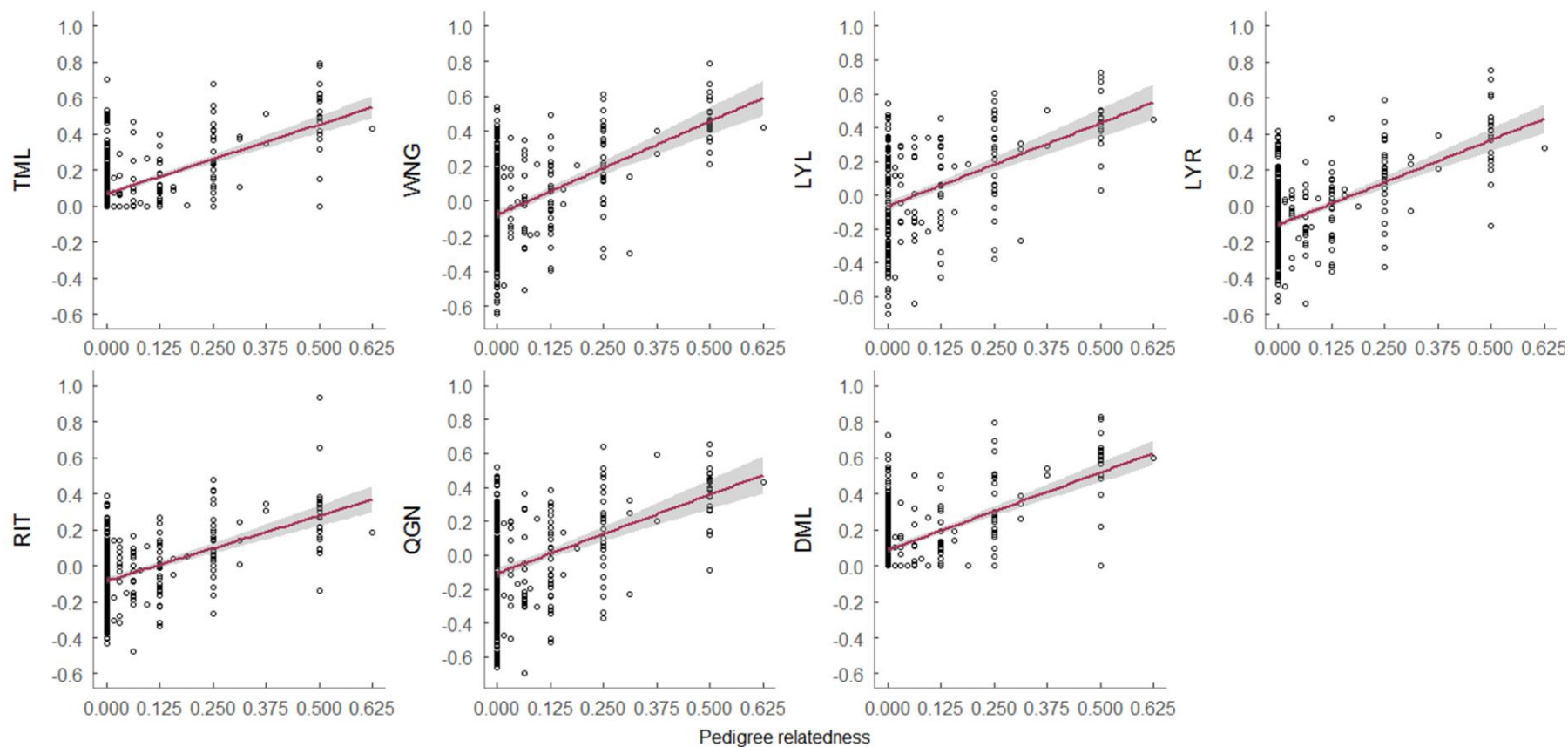


Figure S1 Scatterplots and regression lines for each genetic estimator tested against pedigree relatedness in the empirical dataset. Shading indicates the 95% confidence intervals for the regression lines.

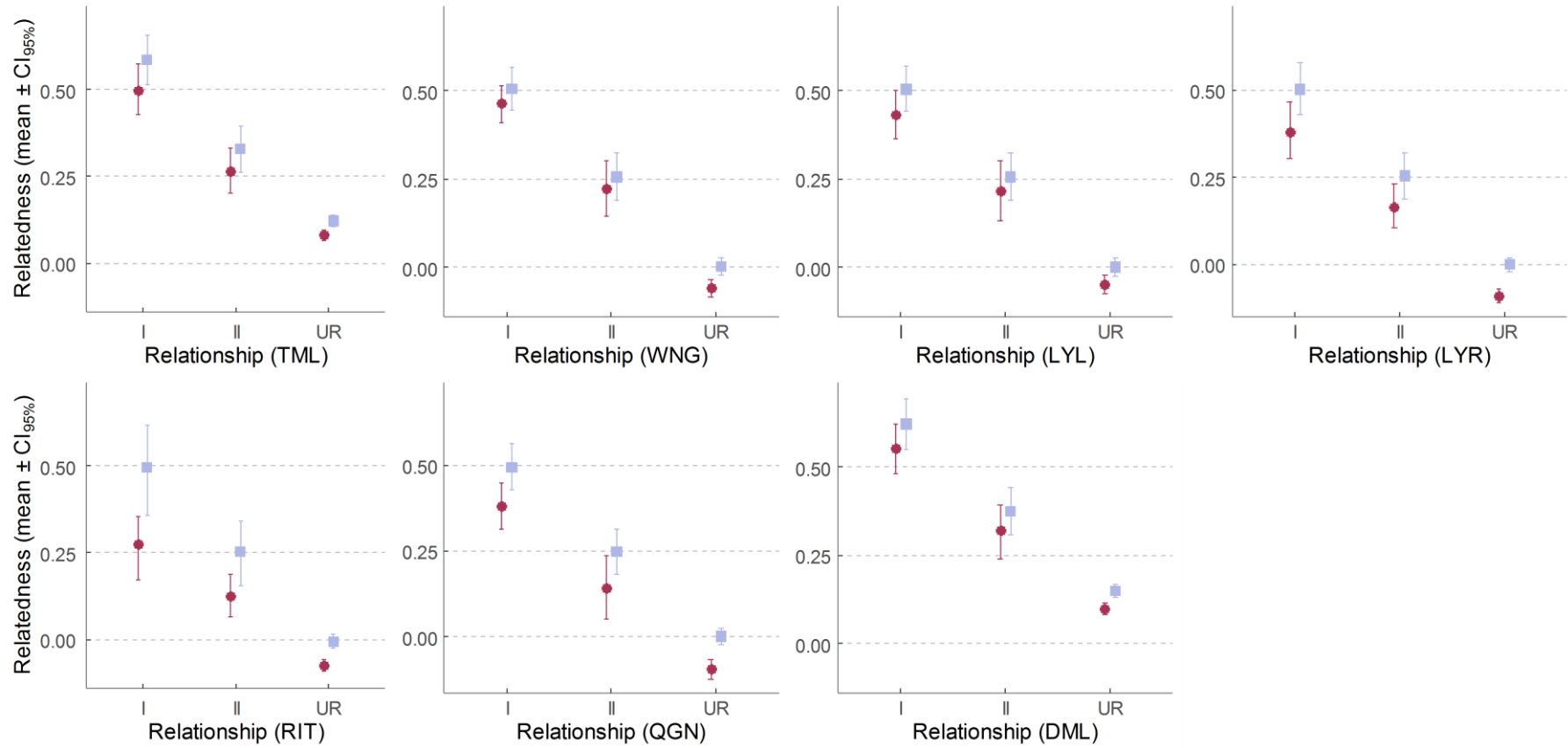


Figure S2 Differences in relatedness values according to known relationships within the empirical dataset (circles) alongside results from the simulated dataset. The graph shows 95% confidence intervals for the means between each relationship category which all differed significantly ($p < 0.0005$) from one another. **I**: First order relationships (relationship coefficient ≥ 0.50); **II**: Second order relationships (relationship coefficient ≥ 0.25) and **UR**: Unrelated individuals (all individuals having a theoretical relationship coefficient below 0.25).

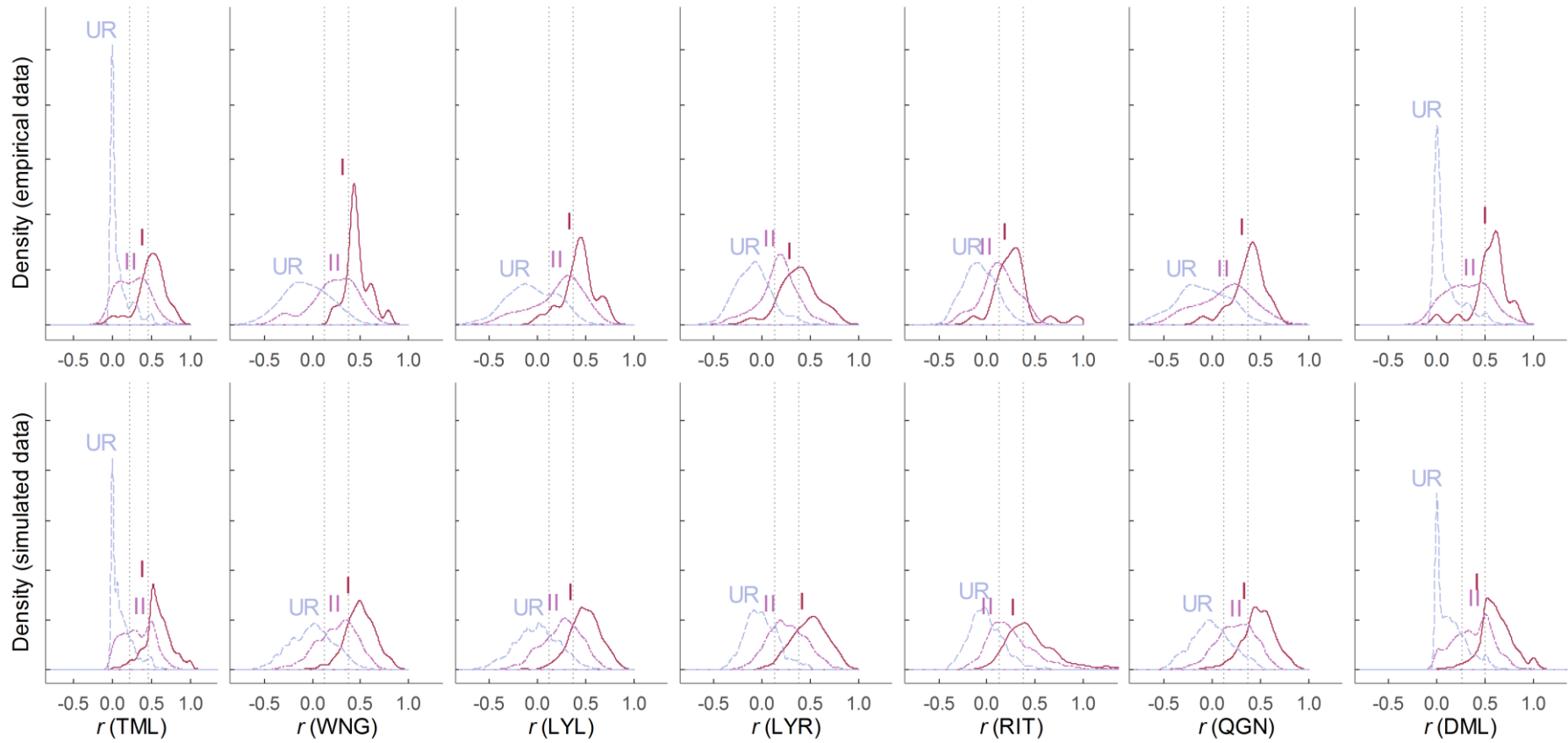


Figure S3 Density plots showing the distribution of relatedness estimates for first degree (I) relatives, second degree (II) relatives and unrelated (UR) pairs of individuals for both the empirical and simulated datasets and using all estimators tested. Vertical dashed lines indicate the midpoints between means used for classification of individuals into relationship categories. Density data was similar for both empirical and simulated data across all estimators.

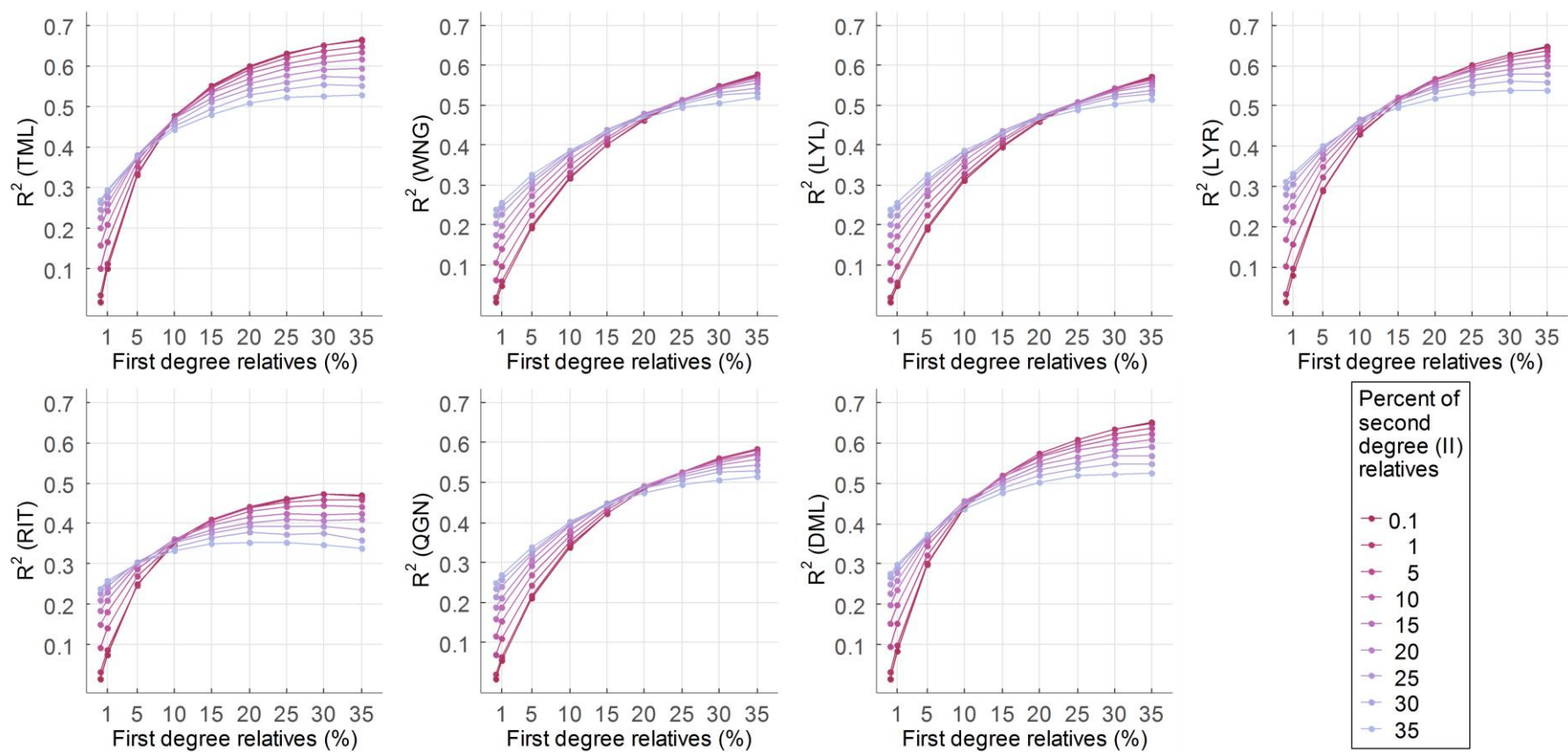


Figure S4 Variation in R^2 with changing proportions of population relatedness. R^2 values represent the mean of 100 replicates at each of the 81 population compositions. Standard errors for the means ranged from 0.0003 to 0.0034

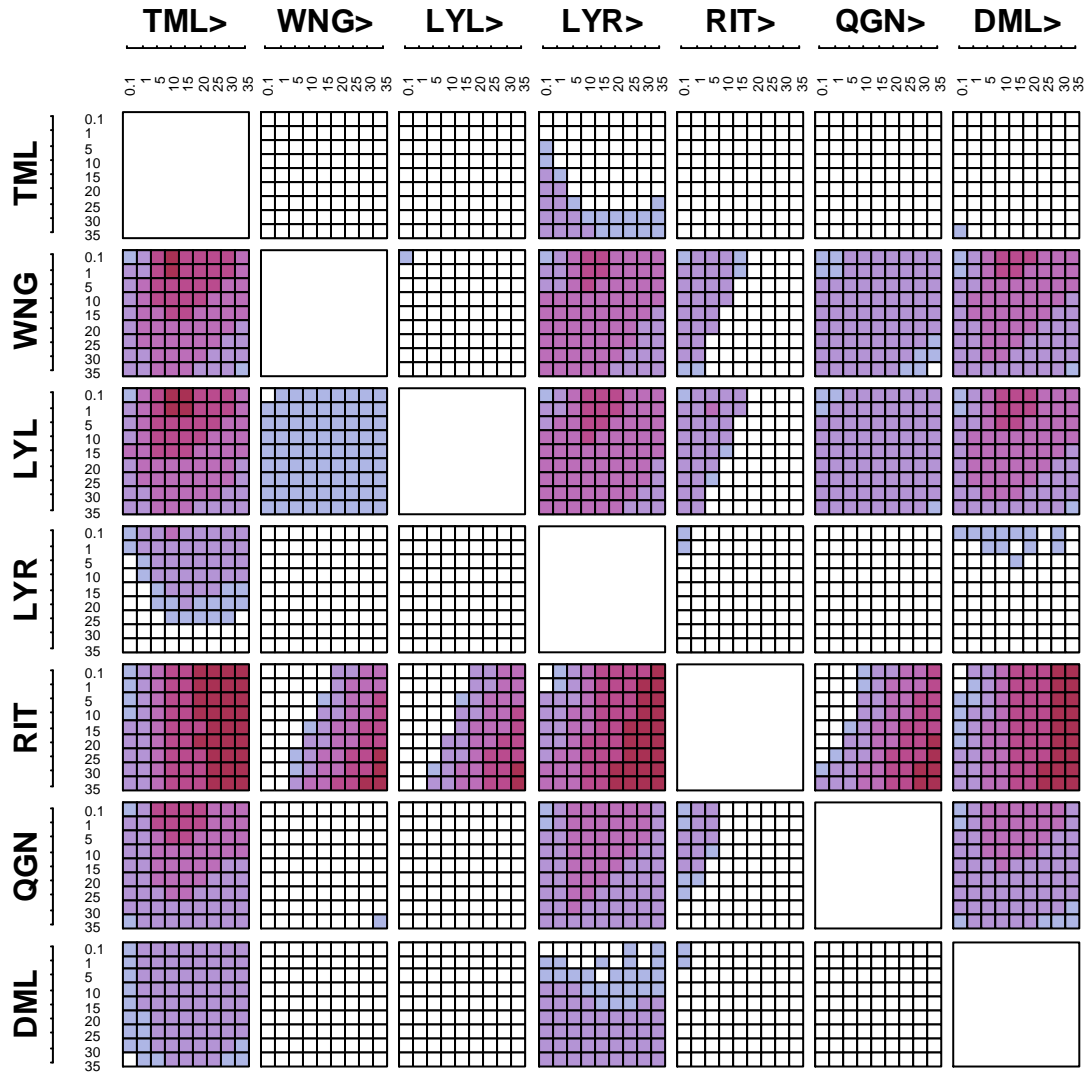


Figure S5 Comparison of R^2 between all estimators tested across 81 different relatedness compositions using allele frequencies from the South Gippsland koala population. The performance of estimators in columns are compared to those in rows. Comparison of each pair of estimators is made up of a 9 x 9 matrix representing the 81 population compositions tested. Columns (from left to right) of each matrix represent the proportion of first degree relatives (0.1, 1, 5, 10, 15, 20, 25, 30 and 35%) while rows (from top to bottom) indicate the proportion of second degree relatives (0.1, 1, 5, 10, 15, 20, 25, 30 and 35%) in the population. Shaded matrix cells indicate that the corresponding column estimator had a higher R^2 than the corresponding row estimator at the particular population composition. Unshaded cells indicate that the column estimator had a lower R^2 value than the row estimator. Different levels of shading indicate the magnitude of the increase in R^2 at five levels: <1% (lightest shading), 1-5%, 5-10%, 10-15% and >15% (darkest shading).

Table S1 Percent of pairwise relationships misclassified by category for the empirical and simulated datasets. Results for the empirical data are shown on the top line of each row, while results for the simulated data are shown on the second line of each row in italics and parentheses. Bold values indicate the best performing estimator in each category for both the empirical and simulated data.

Relationship		TML (%)	WNG (%)	LYL (%)	LYR (%)	RIT (%)	QGN (%)	DML (%)
Known	Assigned							
I	II	0.529	0.529	0.794	1.32	1.85	0.794	0.529
		<i>(0.841 ± 0.047)</i>	<i>(1.08 ± 0.045)</i>	<i>(1.18 ± 0.047)</i>	<i>(1.43 ± 0.050)</i>	<i>(2.15 ± 0.061)</i>	<i>(1.10 ± 0.043)</i>	<i>(0.966 ± 0.055)</i>
I	UR	0.529	0	0.265	0.265	0.265	0.265	0.265
		<i>(0.235 ± 0.028)</i>	<i>(0.0661 ± 0.012)</i>	<i>(0.0926 ± 0.017)</i>	<i>(0.175 ± 0.020)</i>	<i>(0.378 ± 0.033)</i>	<i>(0.156 ± 0.021)</i>	<i>(0.209 ± 0.026)</i>
II	I	2.91	3.17	3.7	2.65	2.65	2.65	3.44
		<i>(2.91 ± 0.066)</i>	<i>(2.61 ± 0.065)</i>	<i>(2.53 ± 0.068)</i>	<i>(2.61 ± 0.071)</i>	<i>(2.34 ± 0.061)</i>	<i>(2.78 ± 0.065)</i>	<i>(2.87 ± 0.062)</i>
II	UR	3.44	1.85	2.65	1.85	2.38	2.65	3.17
		<i>(3.04 ± 0.053)</i>	<i>(2.17 ± 0.052)</i>	<i>(2.16 ± 0.050)</i>	<i>(2.42 ± 0.051)</i>	<i>(3.05 ± 0.057)</i>	<i>(2.27 ± 0.048)</i>	<i>(2.75 ± 0.052)</i>
UR	I	3.97	4.5	6.61	3.97	4.76	8.47	4.23
		<i>(4.13 ± 0.14)</i>	<i>(4.06 ± 0.15)</i>	<i>(4.19 ± 0.18)</i>	<i>(2.69 ± 0.10)</i>	<i>(2.78 ± 0.12)</i>	<i>(4.41 ± 0.15)</i>	<i>(4.01 ± 0.15)</i>
UR	II	11.1	20.4	21.4	16.4	20.1	21.2	12.7
		<i>(12.1 ± 0.18)</i>	<i>(20.5 ± 0.22)</i>	<i>(20.3 ± 0.29)</i>	<i>(15.7 ± 0.24)</i>	<i>(15.2 ± 0.29)</i>	<i>(18.3 ± 0.22)</i>	<i>(14.1 ± 0.18)</i>

Table S2 Changes in r^2 due to missing data at the four least polymorphic loci

Estimator	12 loci	11 loci	10 loci	9 loci	8 loci
TML	38.9	38.9	38.1	34.9	32.2
WNG	33.8	34	33.9	32.5	31
LYL	25.7	26.3	26.3	25.1	23.6
LYR	37.2	37.1	37.1	35.3	34
RIT	22.5	24.6	25.4	24.5	23.9
QGN	22.1	24.4	25	24.1	23.2
DML	39.8	38	37.7	34.4	29.1

Least polymorphic loci removed

All 12 loci: Individuals with complete 12 marker genotypes were used ($n=21$)

11 loci: Phc13 omitted

10 loci: Phc13 and Pcv31 omitted

9 loci: Phc13, Pcv31 and Pcv24.2 omitted

8 loci: Phc13, Pcv31, Pcv24.2 and Pcv25.2 omitted

Table S3 Changes in r^2 due to missing data at the four most polymorphic loci

Estimator	12 loci	11 loci	10 loci	9 loci	8 loci
TML	38.9	26.1	18.7	12.6	9.79
WNG	33.8	22.6	18	11.6	9.82
LYL	25.7	19	14.9	10.3	8.72
LYR	37.2	26.7	20.8	16.5	13.8
RIT	22.5	18.2	15.5	12.4	9.71
QGN	22.1	16.4	13.2	10.3	8.64
DML	39.8	26.6	20.4	14.8	10.1

Most polymorphic loci removed

All 12 loci: Individuals with complete 12 marker genotypes were used ($n=21$)

11 loci: K2.1 omitted

10 loci: K2.1 and Pcv6.3 omitted

9 loci: K2.1, Pcv6.3 and Pcv2 omitted

8 loci: K2.1, Pcv6.3, Pcv2 and K2.1 omitted

Table S4 Overall rate of correctly classified pairs of individuals with data missing for the four least polymorphic loci.

Estimator	12 loci	11 loci	10 loci	9 loci	8 loci
TML	77.6 ± 2.9	76.2 ± 2.9	74.3 ± 3.0	74.8 ± 3.0	74.8 ± 3.0
WNG	71.4 ± 3.1	71.4 ± 3.1	71.9 ± 3.1	70.0 ± 3.2	71.4 ± 3.1
LYL	66.7 ± 3.3	65.2 ± 3.3	61.0 ± 3.4	65.2 ± 3.3	61.9 ± 3.4
LYR	73.3 ± 3.1	73.8 ± 3.0	74.8 ± 3.0	73.3 ± 3.1	72.9 ± 3.1
RIT	64.8 ± 3.3	66.7 ± 3.3	66.7 ± 3.3	63.8 ± 3.3	66.2 ± 3.3
QGN	64.8 ± 3.3	62.4 ± 3.4	63.3 ± 3.3	63.3 ± 3.3	64.3 ± 3.3
DML	78.1 ± 2.9	75.7 ± 3.0	76.2 ± 2.9	73.8 ± 3.0	73.8 ± 3.0

Least polymorphic loci removed

All 12 loci: Individuals with complete 12 marker genotypes were used ($n=21$)

11 loci: Phc13 omitted

10 loci: Phc13 and Pcv31 omitted

9 loci: Phc13, Pcv31 and Pcv24.2 omitted

8 loci: Phc13, Pcv31, Pcv24.2 and Pcv25.2 omitted

Table S5 Overall rate of correctly classified pairs of individuals with data missing for the four most polymorphic loci.

Estimator	12 loci	11 loci	10 loci	9 loci	8 loci
TML	77.6 ± 2.9	72.9 ± 3.1	69.0 ± 3.2	67.6 ± 3.2	66.7 ± 3.3
WNG	71.4 ± 3.1	61.9 ± 3.4	62.9 ± 3.3	57.6 ± 3.4	57.1 ± 3.4
LYL	66.7 ± 3.3	64.3 ± 3.3	59.5 ± 3.4	59.5 ± 3.4	57.1 ± 3.4
LYR	73.3 ± 3.1	69.0 ± 3.2	64.3 ± 3.3	63.3 ± 3.3	62.9 ± 3.3
RIT	64.8 ± 3.3	62.4 ± 3.4	60.5 ± 3.4	61.9 ± 3.4	59.0 ± 3.4
QGN	64.8 ± 3.3	59.5 ± 3.4	59.0 ± 3.4	59.5 ± 3.4	55.7 ± 3.4
DML	78.1 ± 2.9	73.3 ± 3.1	66.7 ± 3.3	66.2 ± 3.3	65.7 ± 3.3

Most polymorphic loci removed

All 12 loci: Individuals with complete 12 marker genotypes were used ($n=21$)

11 loci: K2.1 omitted

10 loci: K2.1 and Pcv6.3 omitted

9 loci: K2.1, Pcv6.3 and Pcv2 omitted

8 loci: K2.1, Pcv6.3, Pcv2 and K2.1 omitted

Chapter 5 | foreword

A large number of DNA extraction kits are commercially available, although the performance of particular kits may vary, especially when isolating DNA from complex biological materials such as scats. Chapter two established that a single DNA isolation kit (Qiagen QIAamp® DNA stool mini kit) performed sufficiently well, delivering DNA isolates providing reliable genetic data. Being constrained to a single DNA isolation kit can, however, be problematic for a project in the event of product unavailability, or discontinuations that may occur from time to time.

This situation occurred part way through this study when the Qiagen QIAamp® DNA stool mini kit became unavailable in Australia. The sudden absence of this kit, known to perform sufficiently well, was a major setback for the project and temporarily prevented the processing of samples. The identification of alternative kits that would perform comparably or better than the Qiagen QIAamp® DNA stool mini kit therefore became a necessity.

Three commercial DNA isolation kits were compared to the Qiagen QIAamp® DNA stool mini kit. Since DNA quantity and quality can also vary greatly between samples from the same individual, samples were pooled and evenly distributed between treatments (different kits). Performance of DNA isolates was compared using DNA quantitation, standard PCR and electrophoresis (band brightness), real time PCR (cycle thresholds) and replicate genotyping using capillary electrophoresis (rates of amplification success and peak heights).

Chapter five identified one alternative kit (Axygen® AxyPrep™ MAG Soil, Stool and Water DNA Kit) which performed better than the Qiagen QIAamp® DNA Stool Mini Kit, therefore DNA isolation from koala scats continued using the Axxygen kit.



Chapter 5

Isolating DNA sourced non-invasively from koala scats: a comparison of four commercial DNA stool kits



Submitted to *Conservation Genetics Resources*



Chapter 5

Isolating DNA sourced non-invasively from koala scats: a comparison of four commercial DNA stool kits

Abstract

Genetic sampling from faeces is a useful method for obtaining DNA samples non-invasively. The quantity and quality of DNA isolated from faecal samples is, however, an important factor affecting the success of downstream analyses. Commercial DNA isolation kits offer an efficient and convenient means for recovering DNA, but the kit methodology can influence the quantity and quality of DNA obtained. Comparisons of kit performance for the isolation of DNA from non-invasive sources for ecological studies based on genetic analysis are uncommon in the literature.

This study compared the quantity and quality of DNA isolated from surface washings of fresh koala (*Phascolarctos cinereus*) faecal pellets (scats) using four commercial DNA isolation kits: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit (AX), Bioline ISOLATE Fecal DNA Kit (BL), Qiagen QIAamp® Fast DNA Stool Mini Kit (QFS), and Qiagen QIAamp® DNA Stool Mini Kit (QS). DNA quantitation, standard PCR and electrophoresis, real time PCR and replicate genotyping using capillary electrophoresis were used to compare the performance of resultant DNA isolates.

The performance of DNA isolated from koala scats varied substantially with the DNA kit utilised. All kits provided accurate genotypes but with differing amounts of missing data. Overall, kit AX performed best, providing DNA isolates of higher quantity and quality compared to kit QS, which has previously been thoroughly assessed for genotyping reliability

using DNA from koala scats. Given the high variability noted, assessing kit performance is an important way to maximise data quality from non-invasively sourced DNA.

Introduction

Non-invasive genetic sampling can provide valuable data for the study of wild animal populations and may offer numerous benefits over samples sourced invasively, such as the ability to obtain greater sample sizes from across large geographic areas which is particularly useful when the species is elusive and/or at low densities and is widely distributed (Piggott & Taylor 2003b; Beja-Pereira *et al.* 2009). In some cases, non-invasively sourced DNA may be the only viable means of obtaining particular population data (e.g. Walker *et al.* 2008). Non-invasive genetic sampling may be limited however, by factors such as reduced DNA quantity and quality and the co-isolation of compounds (PCR inhibitors) that may interfere with molecular analyses (Taberlet *et al.* 1996; Piggott & Taylor 2003a; Beja-Pereira *et al.* 2009). Pilot studies are very important for genetic studies which use non-invasive genetic sampling. Optimising methods to maximise the quantity and quality of target DNA from the sample is vital to ensure the accuracy of genotypic data and integrity of the final results.

The quantity and quality of DNA obtained from non-invasive sources may be affected by many factors dependant on the species of interest (Beja-Pereira *et al.* 2009). A wide range of differing components inhibiting PCR are also likely be present in DNA isolates, depending on the species and sample type. The best methods for isolating DNA from non-invasive sources for a particular species or sample type may not, therefore, be transferrable to other species or sample types (Beja-Pereira *et al.* 2009). As a result, methods need to be individually assessed for the sample type and species of interest (Piggott & Taylor 2003a). In the case of scats, DNA may be affected by environmental conditions, age (Brinkman *et al.* 2010), methods of sampling (e.g. homogenisation, surface scraping, surface washing) and storage methods (Piggott & Taylor 2003a). A large amount of literature evaluating the effect of scat collection, storage and sampling on the isolation of host genomic DNA in non-invasive genetic sampling studies (Luikart *et al.* 2008; Brinkman *et al.* 2010) exists. However, there are very few studies

that evaluate differences between commercial DNA isolation kits for isolation of DNA from non-invasive sources (e.g. Pearson *et al.* 2015; Kranzfelder *et al.* 2016) and even fewer that specifically relate to the comparison of DNA kits for the isolation of faecal DNA from mammals (e.g. Piggott & Taylor 2003a).

Commercial kits are available for isolating DNA from a range of specific environmental and biological samples, including soil and water or tissues such as blood, muscle and even exfoliated intestinal cells found in faecal material. Determining the most effective DNA isolation protocol to suit the sample type, and the subsequent downstream DNA analyses, is important in order to maximise data quality and ensure a high level of confidence in the results (Pompanon *et al.* 2005).

Due to dietary factors and differences in biology, samples from different species can vary widely in the amount and type of inhibitory compounds that are co-isolated with DNA from scats (Piggott & Taylor 2003a; Broquet *et al.* 2007). The performance of different commercial DNA kits has the potential, therefore, to vary between different species and sample types. It is thus important to test the performance of DNA isolation methods for the species and sample type in question. In addition, reliance on a single kit for all DNA isolation requirements within a single genotyping project may result in downtime if the chosen kit becomes unavailable, either temporarily or permanently (e.g. in the event of backorders and discontinuations). Use of more than one DNA isolation kit within a genotyping project enables greater flexibility and hence efficiency of workflow.

The expense of isolating DNA using commercial kits may also potentially place limitations on the number of samples processed during a study, and the cost of different DNA kits per sample can vary substantially (e.g. the cost of DNA isolation per sample for the kits examined in this paper ranged from AU\$3.44 to AU\$16.25 per sample; Table 1). Evaluating a range of

available kits in advance can help to reduce project costs, potentially allowing a larger number of samples to be processed within the available budget.

The Qiagen QIAamp® DNA Stool Mini Kit has previously been shown to provide koala (*Phascolarctos cinereus*) DNA (from scat samples up to four weeks old) of sufficient quantity and quality for reliable genotyping (Wedrowicz *et al.* 2013). The focus of this study was to expand the options for isolating DNA from koala scats by identifying DNA extraction kits that would perform comparably or better than the Qiagen QIAamp® DNA Stool Mini Kit tested by Wedrowicz *et al.* (2013). We therefore aimed to assess the performance of a selection of commercial DNA isolation kits by comparing their performance using fresh koala scat samples. Four commercial kits developed specifically for the isolation of DNA from stool samples were assessed: 1) Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit (AX); 2) Bioline ISOLATE Fecal DNA Kit (BL); 3) Qiagen QIAamp® Fast DNA Stool Mini Kit (QFS) and 4) Qiagen QIAamp® DNA Stool Mini Kit (QS). The performance of kits was compared using measures of DNA quantity and quality including fluorometric quantification of total DNA (target and foreign DNA), quantitative PCR (target DNA copy number) and standard PCR, using both standard agarose and capillary electrophoresis.

Table 1 Comparison of the four commercial DNA kits used to isolate DNA from koala scats in this study.

DNA isolation kit	Abbreviation	Alterations to the manufacturers' protocol	Cell lysis	Inhibitor removal	DNA binding	Approximate cost per sample (pack quantity) in 2015
Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit (cat. no. MAG-STL-M)	AX	None	Lysis buffer with bead beating for 5 mins (chemical/mechanical)	Precipitation	Magnetic beads	AU\$16.25 (100)
Bioline ISOLATE Fecal DNA Kit (cat. no. BIO-52038)	BL	650 µL lysis buffer	Lysis buffer with bead beating for 3 mins (chemical/mechanical)	Silica based filtration column	Silica membrane column	AU\$3.55 (100)
Qiagen QIAamp® Fast DNA Stool Mini Kit (cat. no. 51604)	QFS	Cell lysis (incubation for 1 hour at 35°C)	InhibitEX lysis buffer (chemical)	InhibitEX buffer (inhibitEX and cell lysis buffer from kit QS combined)	Silica membrane column	AU\$11.14 (50)
Qiagen QIAamp® DNA Stool Mini Kit (cat. no. 51504)	QS	Cell lysis (incubation for 1 hour at 35°C)	Buffer ASL lysis buffer (chemical)	InhibitEX tablets	Silica membrane column	AU\$9.70 (50)

Methods

Sample collection

Genotyping reliability has previously been assessed for DNA from fresh koala scats and scats aged under natural conditions for up to four weeks' time (Wedrowicz *et al.* 2013). In this study fresh scats were used to compare the performance of DNA isolation kits. Six scats (<24 hours old) were collected from three individual koalas (K1–K3: 18 scats in total) at the Southern Ash Wildlife Shelter (SAWS), Rawson, Victoria in 2014 (Fig. 1a). Scats were collected using toothpicks and stored at ambient temperature until surface washing (Wedrowicz *et al.* 2013), which was carried out on the same day as collection (~ 5 hours after collection). The surface of each scat was washed in 2 mL of PBS buffer by rolling for 8 mins (Wedrowicz *et al.* 2013). Surface washes from the six scats from each individual were combined and homogenised, then distributed evenly between six 2 mL microfuge tubes so that starting material in each aliquot was approximately representative of the amount that would be obtained from a single scat. Four of the six surface wash aliquots from each individual were randomly allocated to one of the four kits AX, BL, QFS or QS and stored at –20°C until isolation. The remaining two surface washes from each individual were held in reserve. All DNA isolations were carried out within one week of sample collection. Short term storage of surface washes at –20°C was determined to have no effect on DNA quantity and quality (Supporting information, Fig. S1, Table S1). Samples distributed between treatments could therefore be considered approximately equal.

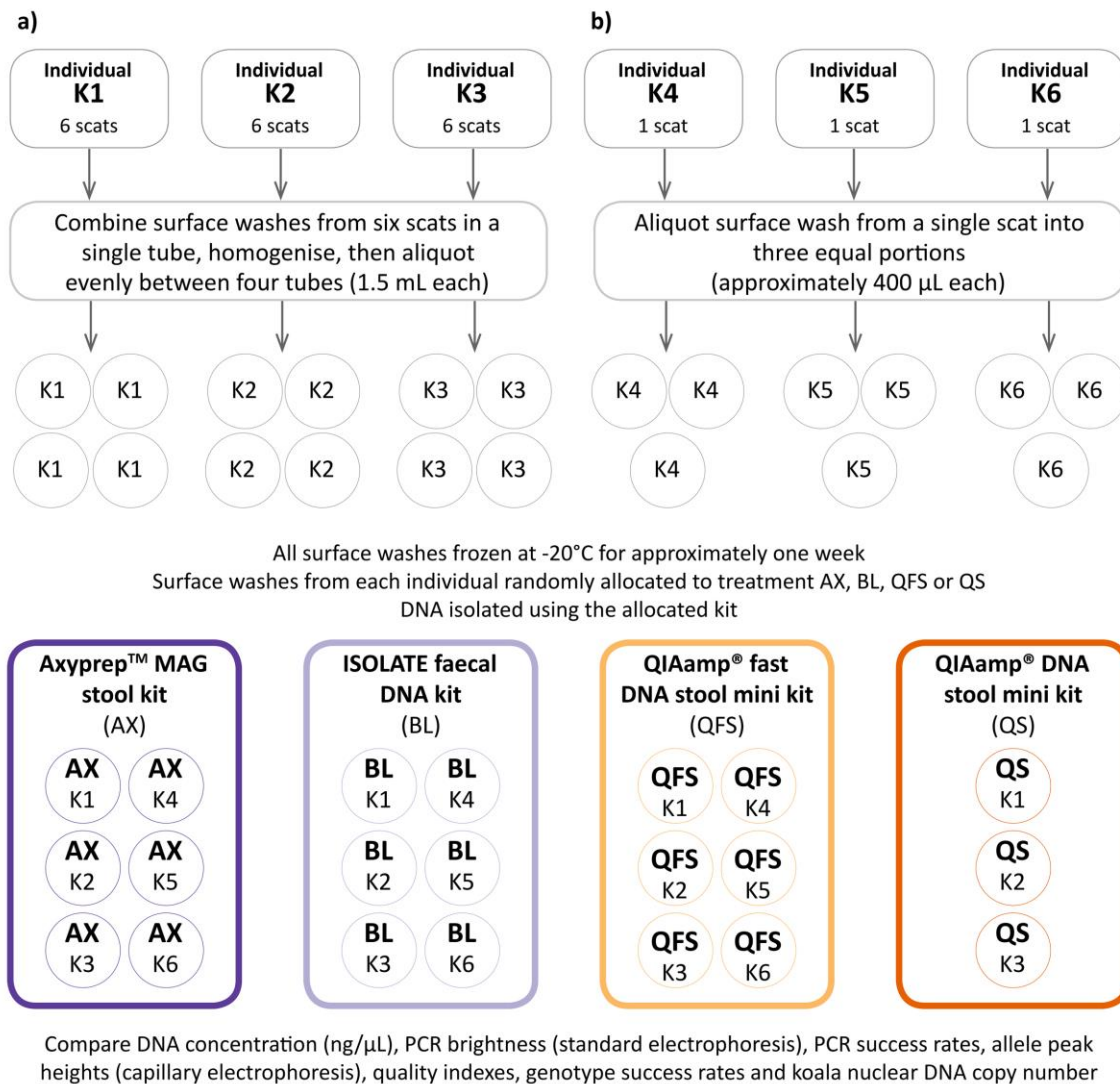


Figure 1 Method used to compare performance of four DNA kits. Kit QS was unavailable at the time that this study was undertaken and laboratory stock of kit QS became depleted during this study. Individuals K4 to K6 were therefore not tested using kit QS.

Wedrowicz *et al.* (2013) use a single scat for DNA isolation, guaranteeing that DNA is isolated from a single koala (as a pose to pooled samples where there is a chance that scats from more than one individual are combined). Although confident that the multiple scats collected as described above were from a single individual, surface washes were also obtained from single scats for three different individuals (K4–K6). Each surface wash was divided into

three equal portions (~ 400 µL) and DNA isolated using only three of the four kits (AX, BL or QFS, Fig. 1b), as the supply of kit QS had been depleted and new stock was unavailable at the time of the study. Dividing surface washes from single scats (for individuals K4–K6) ensured that isolated DNA was from a single koala and was also representative of scats providing lower amounts of starting material (which were a third of that typically obtained from one scat).

DNA isolation

Surface washes were allowed to thaw at room temperature and briefly vortexed. To reduce the volume of surface washes for DNA isolation, concentrate cells available in the wash and ensure consistency in the quantity of starting material between kits, all surface washes were centrifuged at 2500 g for 5 mins. A large proportion of the supernatant was then discarded by pipette so that approximately 50 µL of supernatant was left behind along with the pellet. The pellet and 50 µL of remaining supernatant were re-homogenised by vortexing at low speed for about 10 s (concentrated surface wash). DNA was recovered from approximately equivalent concentrated surface washes from each individual using one of AX, BL, QFS or QS for samples K1–K3 or AX, BL or QFS for samples K4–K6 according to the manufacturers' instructions with slight modifications as outlined in Table 1. For both Qiagen kits, one hour incubations were carried out for cell lysis as per Wedrowicz *et al.* (2013). Steps to minimise the chance of sample contamination included the use of separate laboratories for DNA isolation, PCR setup and post PCR analysis and the use of filter pipette tips. To monitor for contamination, negative controls were included for all PCR experiments.

DNA quantity and quality

DNA quantity and quality were assessed by comparing 1) total DNA (koala and foreign) isolated and 2) the ability to amplify koala DNA using a) real time PCR, b) standard PCR

amplification brightness using agarose gel electrophoresis and c) PCR success rates (PCR+) and mean allele peak heights (APH) using capillary electrophoresis. The quality index (QI) as described by Miquel *et al.* (2006) was also calculated for each sample and used to compare treatments. Additionally, kits were compared using the average number of errors observed in replicate data for each sample (genotyping errors, GT errors) and the number of loci successfully amplified and scored (genotype success, GEN+), all of which are detailed below.

Total DNA yield

Total DNA yield (koala and foreign) was quantified using the high sensitivity double-stranded DNA (HS-dsDNA) assay on the Qubit® 2.0 fluorometer (Life Technologies). Assays were carried out using 1 µL of DNA isolate as per the manufacturer's instructions.

Amplification of koala DNA

Real time PCR was utilised in order to compare amplification performance between different kits by estimating copy number for genomic koala DNA. PCR amplification and therefore copy number estimates are also likely to be influenced by the presence of PCR inhibitors. Copy numbers therefore provided an estimate of amplification ability in the presence of the PCR inhibitors associated DNA isolates from a particular kit. Copy number was estimated using real time primers developed by Markey *et al.* (2007) which targeted an 82 base pair stretch of the koala β-actin gene. Standards were prepared fresh using purified β-actin PCR product for which DNA concentration was estimated by triplicate fluorometric assays and copy number calculated according to the following equation:

$$\text{Copy number (molecules/}\mu\text{L)} = [\text{DNA}] \text{ ng/}\mu\text{L} \times (10^{-9} \text{ g/ng}) \times \\ (660 \text{ g/mol.bp} \times 82 \text{ bp})^{-1} \times (6.022 \times 10^{23} \text{ molecules/mol})$$

Serial dilutions of PCR product were used to produce standards with copy numbers of 10^4 , 10^3 , 10^2 , 10^1 and 10^0 . Assay reactions consisted of 10 μ L of 2X SYBR[®] Select Master Mix (Applied Biosystems) and 0.25 μ M of forward and reverse β -actin primers (Markey *et al.* 2007) made up to 20 μ L with water. Samples and standards were tested in duplicate. Reactions were carried out using an Applied Biosystems StepOnePlus[™] Real-Time PCR System using an initial step of 95°C for 20 seconds (AmpliTaq[®] Fast DNA Polymerase UP activation) followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. A dissociation curve was produced to check for non-specific products.

Agarose gel electrophoresis

DNA quality between isolates obtained by different DNA isolation kits was first compared in-house using standard PCR and gel electrophoresis. We performed microsatellite amplification using Pcv2 and Pcv31 (Cristescu *et al.* 2009) and a sexing PCR using primers GpdEx12/GpdEx13R (Loebel *et al.* 1995; Loebel & Johnston 1997) and IMY1/IMY2 (Watson *et al.* 1998). Microsatellites were amplified using 5 μ L of GoTaq[®] Green Master Mix (Promega), 0.5 μ M of forward and reverse primers, 0.1 μ g/ μ L BSA and 1 μ L of DNA template, adjusted with water to a final volume of 10 μ L. Thermal cycling parameters were 2 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing) and 72°C for 30 seconds (extension) and followed by a final extension at 72°C for 5 minutes. Details of the sexing PCR are provided in Wedrowicz *et al.* (2013). The brightness of PCR products was estimated using GelQuant.NET software (biochemlabsolutions.com). Brightness values for both PCRs were then compared between treatments using paired *t*-tests.

Microsatellite genotyping (capillary electrophoresis)

Samples were genotyped by the Australian Genome Research Facility (AGRF), Melbourne, Australia for twelve microsatellite loci: K2.1, K10.1, Pcv2, Pcv6.1, Pcv6.3, Pcv24.2, Pcv25.2, Pcv30, Pcv31 (Cristescu *et al.* 2009), Phc2, Phc4 and Phc13 (Houlden *et al.* 1996b) as outlined in Wedrowicz *et al.* (2013). AGRF carried out PCRs and product separation using capillary electrophoresis. Three replicate genotypes were obtained when all samples from the same individual (across different kits) had a total (koala and foreign) DNA concentration above 1 ng/μL (quantified as described above), while four replicate genotypes were obtained when any of the samples were below the 1 ng/μL threshold. Based on Taberlet *et al.* (1996) and Valière *et al.* (2007), consensus genotypes were constructed using the following rules: 1) alleles had to appear at least twice to be counted; 2) where four replicates were used, homozygous alleles had to appear at least three times; and 3) loci giving ambiguous results were omitted (scored as a failed reaction).

Measures of DNA quality for genotypic data

For genotypic data (12 marker genotypes replicated three or four times) obtained using capillary electrophoresis, *ConGenR* (Lonsinger & Waits 2015) was used to produce consensus genotypes. The error rates estimation calculator in GIMLET v 1.3.3 (Valière 2002) was used to calculate rates of amplification success (PCR+, proportion of loci successfully amplified) and genotyping error (GT errors). Quality indexes (QIs) were calculated by assigning a value to each replicate genotype at a given locus (Miquel *et al.* 2006). A score of one was assigned to the genotype if it matched the consensus genotype, otherwise a score of zero was assigned (whether it differed due to a failed reaction or error; Miquel *et al.* 2006). Genotype success (GEN+) was defined as the number of loci successfully amplified and scored for each consensus genotype. Peak heights obtained from capillary electropherograms were averaged

for each locus to provide average peak height (APH). Samples were paired according to the individual koala from which the surface wash originated for statistical analysis. Differences between means were evaluated using paired *t*-tests in R 3.1.1 (R Core Team 2014). Paired statistical tests comparing kits AX, BL and QFS used samples from six individuals (K1–K6) while comparisons including kit QS compared samples from three individuals (K1–K3). Although sample sizes are relatively small, variations in starting material provided by different scats from the same individual were eliminated by pooling scat surface washes from the same individual and evenly distributing between treatments (Fig. 1), providing direct comparisons between DNA isolated from the same amount of starting material.

Results

The best performing kit for the isolation of koala DNA from scats, across all tests for DNA quantity and quality, was the Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit (kit AX). Kits BL and QFS were found to provide DNA isolates of similar quality to one another but with lower performance compared to kit AX. Kit QS, previously found to produce reliable genotypes using the methods described in Wedrowicz *et al.* (2013), performed slightly better than kits BL and QFS but not as well as kit AX.

Total DNA concentration

The total amount of DNA (koala and foreign) isolated was highest for kit AX (Table 2, $p=0.02$). The AX kit produced DNA isolates with a mean increase of 3.6, 3.5 and 5.0 ng/μL DNA compared to kits BL ($p=0.03$), QFS ($p=0.03$) and QS ($p=0.06$) respectively (Table 3). Differences in mean DNA concentration between kits BL, QFS and QS were smaller and all insignificant ranging from 0.11 to 0.72 ng/μL (Table 3).

Table 2 Summary of kit performance. Average DNA concentrations, mean copy number of koala nuclear DNA per reaction (copy no.), PCR amplification success (PCR+), mean allele peak height (APH), quality index (QI), number of genotyping errors (GT errors) and genotype success (GEN+) for samples from six individuals (K1–K6) divided evenly between three (K4–K6) or four (K1–K3) DNA isolation kits. Standard errors for the means are also shown. Kit QS only considered individuals K1–K3. **AX**: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL**: Bioline ISOLATE Fecal DNA Kit, **QFS**: Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS**: Qiagen QIAamp® DNA Stool Mini Kit.

Kit	Ind (n)	PCRs (n)	DNA (ng/μL)	Copy no.	PCR+ (%)	APH	QI	GT errors	GEN+ (/12)
AX	6	276	4.1 ± 1.4	585 ± 253	92.4 ± 1.6	3633 ± 213	0.90 ± 0.02	0.17 ± 0.17	11.17 ± 0.40
BL	6	276	0.53 ± 0.19	580 ± 390	72.5 ± 2.7	3686 ± 244	0.59 ± 0.03	4.0 ± 1.6	8.83 ± 1.1
QFS	6	276	0.64 ± 0.23	105 ± 44	69.9 ± 2.8	3854 ± 230	0.60 ± 0.03	1.5 ± 1.5	8.17 ± 1.9
QS	3	132	1.3 ± 0.57	169 ± 35	77.3 ± 3.7	3237 ± 255	0.70 ± 0.04	1.3 ± 0.67	9.67 ± 0.88

Table 3 Mean of differences between paired sample averages for DNA concentration (DNA), mean copy number of nuclear koala DNA per reaction (copy no.), PCR success (PCR+), mean allele peak height (APH), quality indexes (QI), number of genotyping errors (GT errors) per sample and genotyping success (GEN+) given in table 2. The direction of the differences are relative to kit 1. Significant differences at or below $p=0.05$ are marked with an asterisk. **AX**: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL**: Bioline ISOLATE Fecal DNA Kit, **QFS**: Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS**: Qiagen QIAamp® DNA Stool Mini Kit.

Kit 1	Kit 2	Ind (n)	DNA (ng/μL)	Copy no.	PCR+ (%)	APH	QI	GT errors	GEN+
AX	BL	6	3.6*	5.58	19.9*	-53	0.32*	-3.8	2.3
AX	QFS	6	3.5*	481	22.5*	-221	0.30*	-1.3	3.0
BL	QFS	6	-0.11	475	2.5	-168	-0.011	-1.0	0.67
AX	QS	3	5.0	395	15.9*	1070*	0.23*	2.5	1.3
BL	QS	3	-0.72	44.1	-18.9*	302	-0.23*	2.3	-2.3
QFS	QS	3	-0.55	-115	-27.3*	665	-0.25*	-1.3	-4.0

Koala nuclear DNA copy number

On average, the amount of nuclear koala DNA isolated using kits AX and BL was similar and both exceeded the copy number isolated by kits QFS and QS (Table 2). Paired *t*-tests showed only kit AX had a significantly higher copy number compared to kit QFS ($p=0.04$). Kit BL had a koala nDNA copy number of 580 ± 390 that did not correspond to increases in other performance measures, potentially due to the high copy number estimated for sample K5 (Supporting information, Fig. S2).

Amplification brightness (gel electrophoresis)

Both DNA concentration and PCR performance using agarose gel electrophoresis of DNA isolated from koala scats were approximately equal using the two Qiagen kits, QFS and QS (Table 2, Fig. 2). Using agarose gel electrophoresis, kits AX and BL tended to produce brighter bands than kits QFS and QS (Fig. 2).

Genotyping PCR success (capillary electrophoresis)

PCR success rates (PCR+) for the genotypic data using 12 microsatellites and three or four replicates were best for kit AX, followed by kit QS, BL and QFS (Table 2). PCR success rates were highest for kit AX (92.4%), which was significantly higher than kits BL, QFS and QS, where PCR success was 72.5%, 69.9% and 77.3% respectively (Tables 2 and 3, $p<0.0005$). Higher amplification rates observed for kit QS compared to kits BL and QFS were also significant (Table 3, $p<0.0005$).

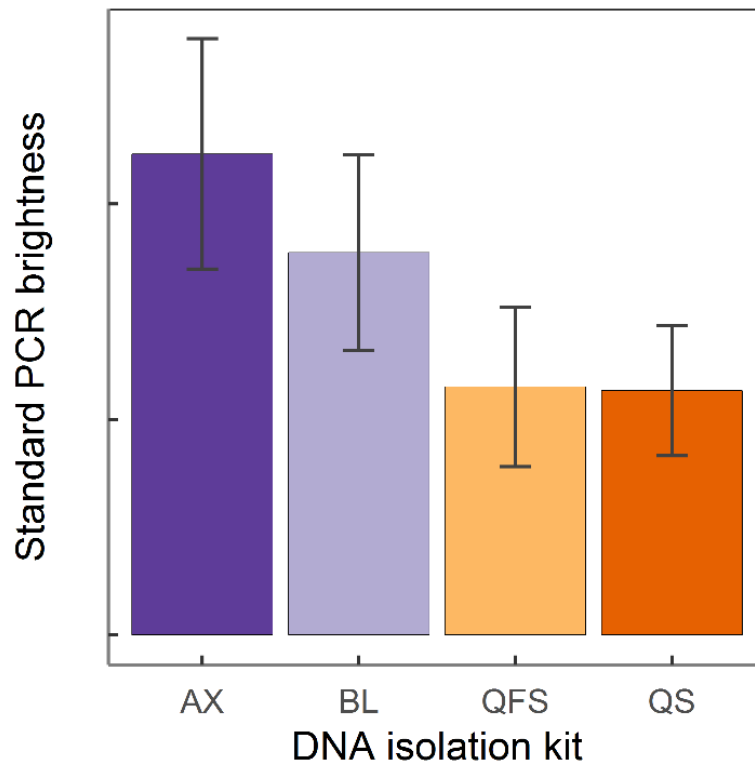


Figure 2 Mean and standard error of amplification brightness determined using agarose gel electrophoresis (2 x Pcv2, 2 x Pcv31 and 1 x XY PCRs) of paired samples (K1–K3) isolated using one of four commercial DNA kits ($n=15$ PCRs for each kit). **AX**: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL**: Bioline ISOLATE Fecal DNA Kit, **QFS**: Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS**: Qiagen QIAamp® DNA Stool Mini Kit.

Genotyping peak height (capillary electrophoresis)

DNA genotyping revealed little difference in mean peak height according to the DNA kit used (Table 3). On average, kit AX yielded over 3 ng/μL more total DNA than kits BL, QFS and QS (significant at 95% level for kits BL and QFS compared to AX), which is likely to reflect an increase in the amount of koala DNA isolated by kit AX. While PCR success for kit AX was greater than for kits BL, QFS and QS (see above), a corresponding increase in APH for kit AX was not observed.

Quality indexes

A comparison of quality indexes between treatments found that kit AX was, again, superior to other kits (Table 2). Kit AX had QI values that were higher by 0.32, 0.30 and 0.23 than kits BL, QFS and QS respectively (Table 3, $p=0.001$). Kit QS had significantly higher QI values than kits BL and QFS ($p=0.03$) while kits BL and QFS had similar QI values (Table 3).

Patterns of performance appeared to vary according to individual (Fig. 3). For example, quality indexes by individual (Fig. 3) followed a similar pattern for individuals K1, K2 and K6 (where the order of performance was AX/QS, BL then QFS) while for individuals K3 and K5 differences between kits were negligible. Differences between pooled samples from different individuals in this study were however not significant.

The QI across loci also varied between kits. Using kit AX, five loci produced maximum QI values of 1.0 (K2.1, K10.1, Pcv6.1, Pcv30 and Phc13) while the remaining five had a mean QI value of 0.96 ± 0.04 (Pcv6.3, Pcv24.2, Pcv25.2, Pcv31 and Phc4). For all kits, most of the reductions in QI resulted from two loci, Pcv2 and Phc2, which produced QI values of 0.65 ± 0.10 and 0.39 ± 0.10 , respectively for kit AX (Fig. 4).

Genotype errors

Kit AX also produced the fewest observed genotypic errors within replicate data, with an average of 0.17 errors per 12 marker genotype compared to 4.0, 1.5 and 1.3 errors per 12 marker genotype for kits BL, QFS and QS, respectively (Table 2).

Genotype success

Genotypes produced by all kits were 100% identical at all available loci for each of the six individuals for which genotypic data were obtained. Kit AX performed best with the average 11.2 of 12 loci successfully amplified and genotyped, compared to 8.8, 8.2 and 9.7 loci out of 12 using kits BL, QFS or QS, respectively (GEN+, Table2). However, the apparent increase in genotyping success for kit AX was not significant when compared to kits BL ($p=0.06$), QFS ($p=0.06$) or QS ($p=0.12$).

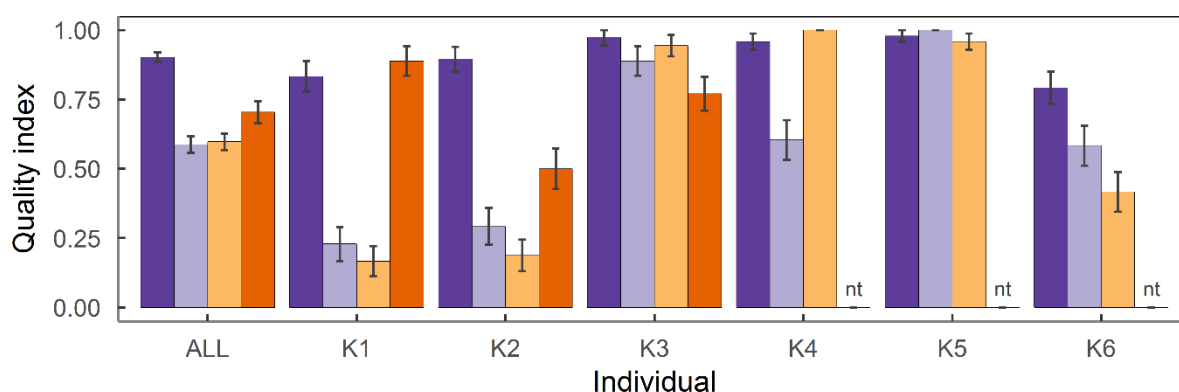


Figure 3 Average quality index by individual and DNA kit used. For individuals K1-K3 four kits were tested (AX, BL, QFS or QS), while for individuals K4-K6, three kits (AX, BL and QFS) were tested. The order of the bars within each group is kit AX (dark purple shading), followed by kit BL (light purple shading), kit QFS (light orange shading) and kit QS (dark orange shading). **nt**: not tested, **AX**: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL**: Bioline ISOLATE Fecal DNA Kit, **QFS**: Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS**: Qiagen QIAamp® DNA Stool Mini Kit.

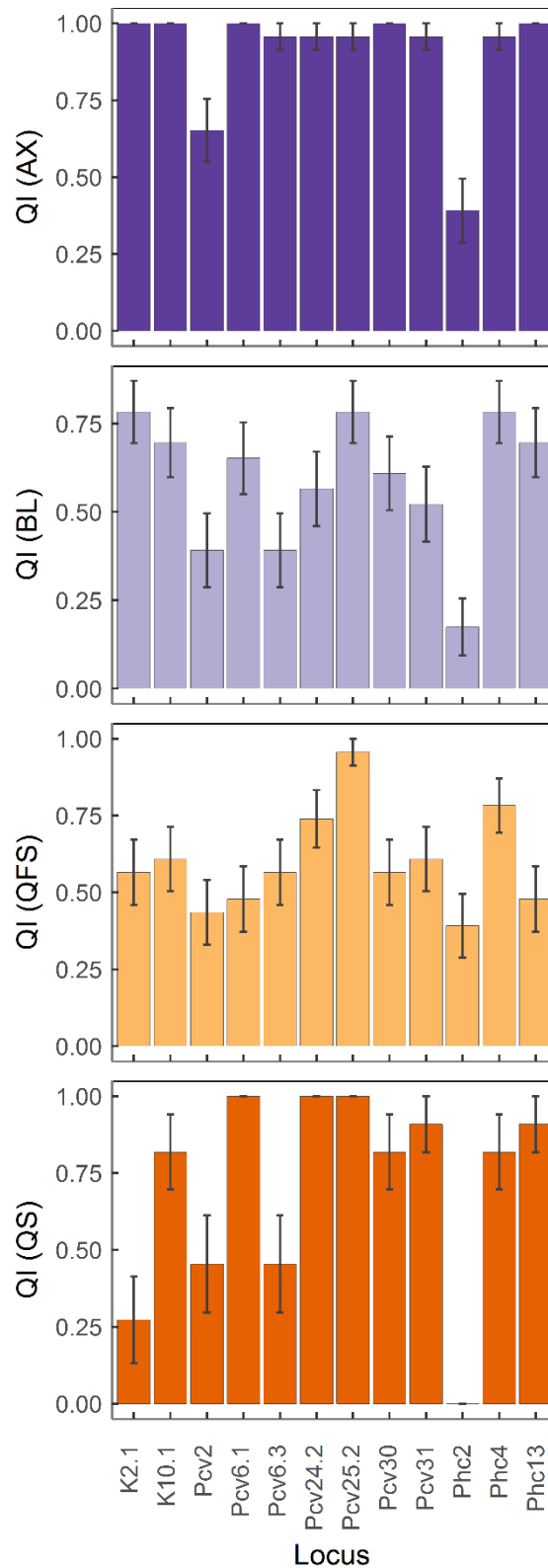


Figure 4 Average quality index for each locus according to the DNA kit used. For kits AX, BL and QFS samples from six individuals (K1-K6) were compared while for kit QS samples from three individuals (K1-K3) were used. **AX:** Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL:** Bioline ISOLATE Fecal DNA Kit, **QFS:** Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS:** Qiagen QIAamp® DNA Stool Mini Kit.

Discussion

The quantity and quality of DNA sourced from scats can vary substantially among samples from the same individual (Piggott & Taylor 2003a; Walker *et al.* 2009) and even between subsamples of the same scat (Stenglein *et al.* 2010), which can make direct comparisons of different collection and storage methods difficult. In this study, DNA isolation protocols were directly compared by homogenising the starting material from numerous samples from the same individual and evenly distributing between treatments. This study showed that the choice of commercial DNA isolation kit has a significant effect on the quantity and quality of genomic DNA obtained from koala scat samples. Overall, the Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit was found to provide DNA isolates from the surface washes of koala scats with the highest quantity and quality of koala DNA.

The performance of DNA isolates varied between individuals in some cases, though these differences were not significant (Fig. 3). This is likely due to the presence of differing inhibitory molecules isolated from scats between individuals (due to dissimilarities in diet and biology) and differences in each kit's ability to remove particular compounds. This experiment was designed to detect differences between DNA isolation kits rather than individuals, so the lack of a statistical difference between individuals is most likely due to the small number of individuals tested in this study.

In general, differences in mean copy number of koala nuclear DNA (Table 3) were similar to differences in mean amplification brightness (Fig. 2), where amplification of DNA isolated using kits AX and BL were brighter than those isolated by kits QFS and QS. A possible explanation for the higher levels of nuclear koala DNA isolated using kits AX and BL compared to kits QFS and QS could be attributed to incomplete lysis of koala cells using buffer based lysis methods (kits QFS and QS) and more efficient cell lysis using bead beating

(kits AX and BL). This could indicate that bead beating is more efficient for lysis of koala intestinal cells than chemical methods alone. Increased performance for DNA isolation methods utilising bead beating compared to chemical lysis methods has also been reported in a study isolating bacterial DNA from faecal samples (Ferrand *et al.* 2014), but bead beating has been associated with greater levels of DNA shearing (Yu & Morrison 2004). Given the high level of amplification success using kit AX, microsatellite genotyping appears to be unaffected by the level of shearing; the largest microsatellite marker used in this study was 319 bp in size (Pcv6.3). Additionally, mitochondrial DNA markers of up to 1500 bp in length were amplified with high success rates using DNA isolated with kit AX (data not shown).

Kit QS has previously been shown to provide reliable genotypes from DNA isolated from koala scats (Wedrowicz *et al.* 2013). All kits tested for this study provided identical consensus genotypes for each individual sampled, albeit with variable numbers of loci with missing data. Kit AX, however, proved to be the best kit for isolating koala DNA from their scats, having the highest mean DNA concentration, koala nuclear DNA copy number, quality indexes, numbers of loci successfully scored in consensus genotypes, levels of amplification success and lowest levels of genotyping error.

Increased error rates may increase the number of replicates required to obtain a consensus genotype with a high level of confidence (Valière *et al.* 2002). Kit QS has been shown to provide reliable consensus genotypes using three or four replicate genotypes (Wedrowicz *et al.* 2013). In this study, only kit AX was found to produce fewer errors than kit QS, indicating that only kit AX (or QS) can be confidently used to extract DNA from koala scats suitable for genotyping with the number of replicates recommended by Wedrowicz *et al.* (2013). Given that the error rate appears higher for kits BL and QFS, the use of these kits would require

further method optimisation and assessment of error rates in order to determine the number of replicates required for reliable genotyping using these kits.

To maximise reliability when isolating koala DNA from scats, we therefore recommend the use of kit AX or QS where possible. The lower error rate observed using kit AX could, potentially, reduce the number of replicates required for reliable genotyping, though a more detailed assessment of the error rates associated with DNA isolated using kit AX would be required.

Kit AX was the most expensive kit tested in this study (Table 1). Given the importance of DNA isolation methods in maximising DNA quantity and quality and determining the performance of downstream DNA analyses, extra costs at the DNA isolation stage may be largely offset by fewer failed reactions and the number of genotypes that might need to be discarded due to poor quality data. There is also potential to reduce genotyping costs if the error rate associated with kit AX is low enough that the number of replicates needed to obtain reliable consensus genotypes can be reduced.

Different patterns of amplification brightness were observed for agarose electrophoresis conducted in house and capillary electrophoresis conducted off-site by AGRF (Fig. 2, Table 2). One potential explanation is that PCR conducted offsite at AGRF did not include BSA, which can facilitate amplification in the presence of PCR inhibitors. To test the effect of BSA we divided samples into two equal portions, adding BSA to one and water to the other. Genotyping of these samples showed that PCR inhibition was impacting on analyses performed by AGRF (Supporting information, Fig. S3). The observed difference may therefore be due to the alleviation of inhibition in the presence of BSA for PCR conducted in-house, along with different levels of inhibitor co-isolated by each kit. Including BSA in

amplifications carried out offsite will therefore further increase success and reliability of the genotyping method used here.

For all kits tested, two microsatellite markers (Pcv2 and Phc2) performed at a lower level than the remaining ten (Fig. 4). Replacing Pcv2 and Phc2 with more reliable markers may be a useful way to further increase the reliability of genotyping from koala scats. This would require screening and replicate genotyping of additional microsatellite markers currently available for the koala (i.e. Houlden *et al.* 1996a; Cristescu *et al.* 2009; Ruiz-Rodriguez *et al.* 2014).

Conclusion

This research highlights the value of carrying out pilot studies to determine the commercial DNA kit that will provide the best quantity and quality of DNA from the target sample. It is important to remember that the relative performance of different commercial DNA isolation kits will differ for different species and types of starting material. The protocol to assess the performance of DNA kits used in this study (Fig. 1) could be readily adapted for other species and biological samples in order to assess and compare DNA quantity and quality between DNA isolation kits.

As demonstrated here, trialling alternate kits, even for established methods, may identify commercial products that will provide increased amounts of DNA with better quality. In addition, kits providing equivalent results can also be identified, which may be useful if a chosen kit becomes temporarily unavailable, or the manufacturer discontinues production.

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Chapter 5 | Supporting information

Storage of surface washes at -20°C

Method

The impact of storing surface washes at -20°C on DNA quantity or quality was tested in two ways. Firstly, surface washes from single scats were divided into two equal portions; DNA was isolated immediately from one, and after two weeks storage at -20°C for the other. For this task, surface washes were performed on six scat samples, each collected from the ground beneath six different koalas (ST01-ST06) at the Southern Ash Wildlife Shelter (SAWS), Rawson, Victoria during 2014. Each of the surface washes was divided into two equal aliquots and randomly allocated to one of two groups. DNA was isolated immediately from one group of surface washes following Wedrowicz et al. (2013). The second group of surface washes was stored at -20°C for two weeks. After two weeks storage, the stored surface washes were allowed to thaw at room temperature and DNA was isolated, again following Wedrowicz et al. (2013). DNA concentrations, standard PCR and agarose gel electrophoresis were used to look for differences in DNA quantity or quality between paired treatments.

Secondly, two separate scats were collected from a further five individuals (ST07-ST11). The two scats from each individual were randomly allocated to one of two groups. DNA was isolated immediately after surface washing for the first group; while the surface washes from the second group were stored at -20°C for three weeks before DNA isolation. Isolated DNA from both groups of scats in this second part were genotyped to determine whether final genotypes were impacted by storage at -20°C .

Surface wash storage results

DNA isolated from fresh or stored (-20°C , two weeks) surface washes did not significantly differ in DNA concentration, PCR success rates or amplification brightness (using standard PCR and agarose gel electrophoresis, Fig. S1).

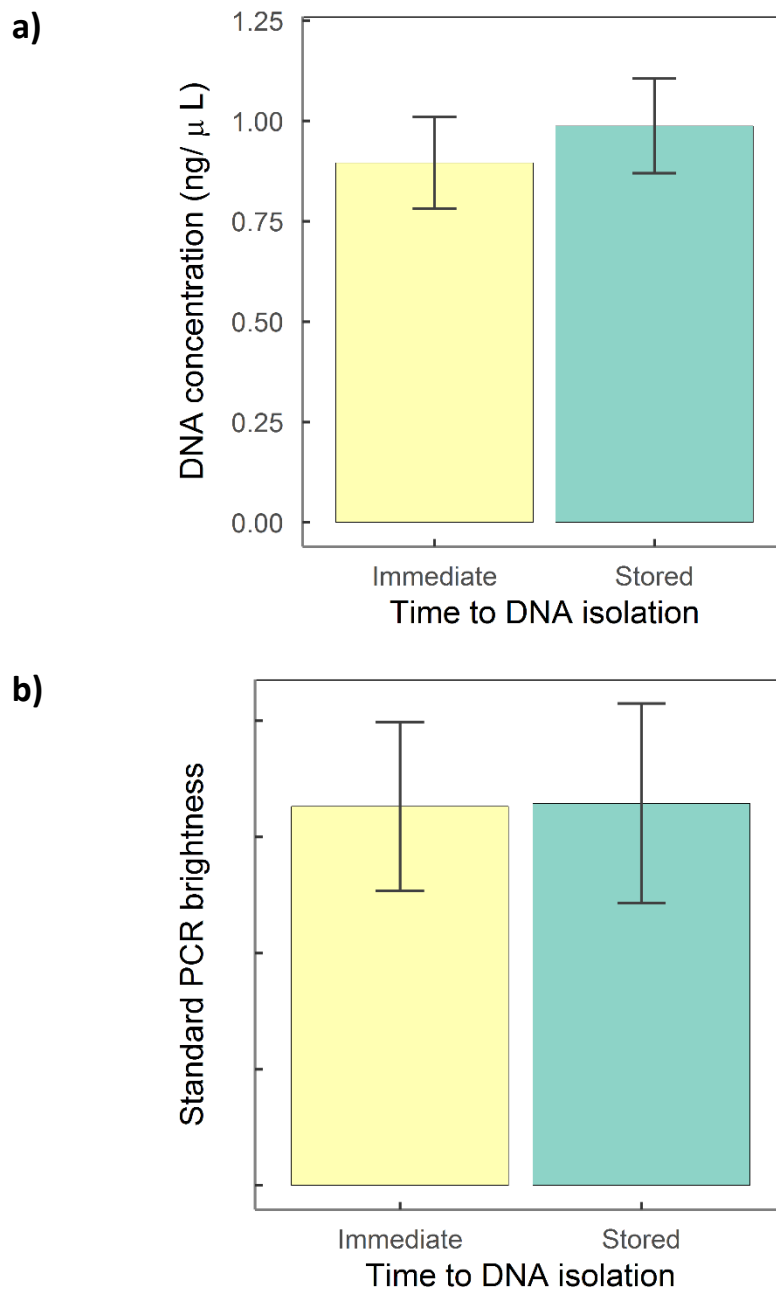


Figure S1 a) Mean DNA concentrations of paired samples (ST01-ST06) from which DNA was isolated either immediately after surface washing or after two weeks storage at -20°C . Error bars represent the standard error for the mean. **b)** Standard PCR brightness using agarose gel electrophoresis (Pcv31 and XY PCRs) of paired samples (ST01-ST06) from which DNA was isolated either immediately after surface washing or after two weeks storage at -20°C .

Genotyping of two separate scats from five individuals (ST07-ST11: one isolated without storing the surface wash, the second isolated from a surface wash stored at –20°C for three weeks) gave identical genotypes in all cases and paired *t*-tests showed no reduction in PCR success rates (Table S1). PCR success and mean allele peak heights of the genotypic data (ST07-ST11) stored at –20°C for three weeks prior to DNA isolation were not reduced (Table S1).

Table S1 Samples genotyped for independent scats from five individuals (ST07–ST11). Number of loci positively amplified and scored and comparison of the number of identical loci between paired samples.

Sample ID	Treatment	Positive loci	Identical loci
ST07	Immediate	12	12/12
	Stored	12	
ST08	Immediate	12	12/12
	Stored	12	
ST09	Immediate	10	10/10
	Stored	10	
ST10	Immediate	11	11/11
	Stored	12	
ST11	Immediate	12	12/12
	Stored	12	
Comparison of mean measures of PCR success			
Variable	Treatment	PCR+(%)	<i>p</i> -value
PCR success	Immediate	88.9	ns (0.996)
	Stored	95.2	
Variable	Treatment	APH	<i>p</i> -value
Average peak height	Immediate	6518	ns (0.546)
	Stored	6861	

ns: not significant

PCR+: amplification success

APH: Mean allele peak height

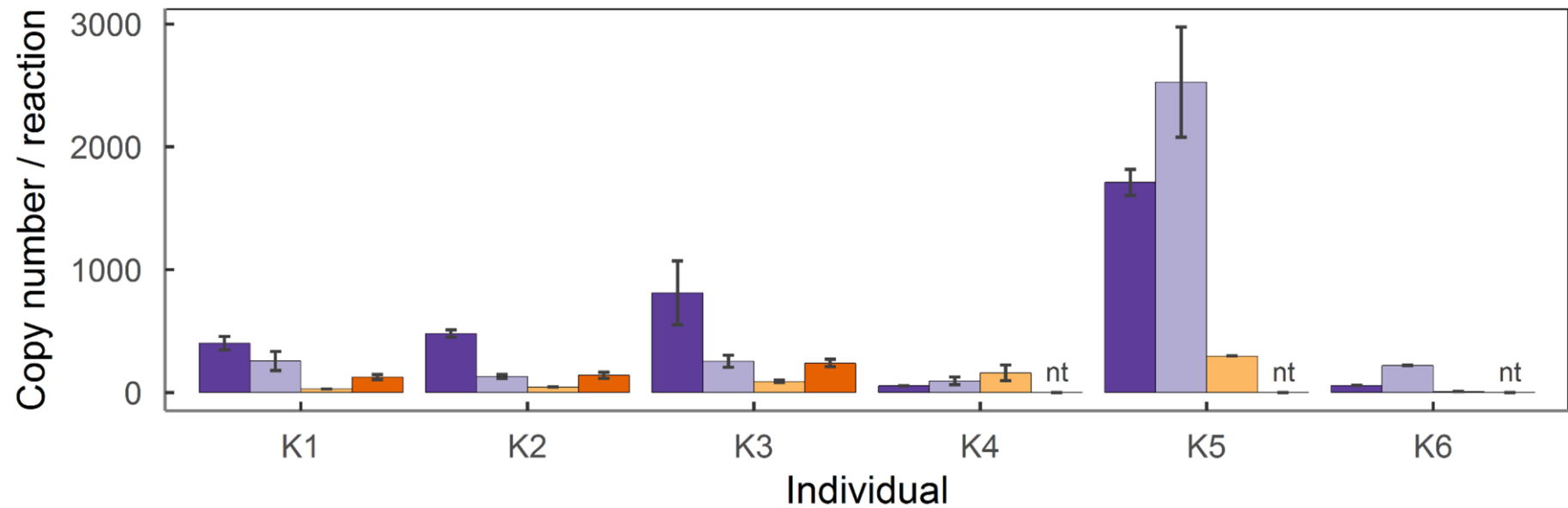


Figure S2 Total number of koala genomic DNA (β -actin) copies per DNA isolate. Error bars represent the standard deviation calculated from replicate reactions. For individuals K1-K3 four kits were tested (AX, BL, QFS or QS), while for individuals K4-K6, three kits (AX, BL and QFS) were tested. Kit AX is represented by dark purple bars, kit BL by light purple bars, kit QFS by light orange bars and kit QS by dark orange bars. **nt**: not tested, **AX**: Axygen® AxyPrep™ MAG Soil, Stool, and Water DNA Kit, **BL**: Bioline ISOLATE Fecal DNA Kit, **QFS**: Qiagen QIAamp® Fast DNA Stool Mini Kit and **QS**: Qiagen QIAamp® DNA Stool Mini Kit, **nt**: not tested.

Addition of BSA to DNA isolates for genotyping

Bovine serum albumin (BSA) is an amplification facilitator that can be used to overcome PCR inhibition. The initial PCR protocol used offsite for genotyping koala DNA isolates did not include BSA, potentially contributing to the differences in results for analyses carried out in house or offsite. The effect of adding BSA to DNA isolates prior to submission for genotyping on amplification was therefore tested after comparing DNA isolation kits.

Five DNA isolates (T1-T5) were separated into two equal aliquots of 45.6 μL . To the first aliquot, 2.4 μL of water was added and to the second aliquot 2.4 μL BSA (20 $\mu\text{g}/\mu\text{L}$; Thermo Scientific cat. no. B14). Each of the two aliquots was genotyped in duplicate using 12 microsatellite markers: K2.1, K10.1, Pcv6.1, Pcv2, Pcv6.3, Pcv24.2, Pcv25.2, Pcv30, Pcv31 (Cristescu *et al.* 2009), Phc2, Phc4 and Phc13 (Houlden *et al.* 1996), resulting in a total of 48 PCRs (24 without BSA and 24 with BSA) for each sample and 120 PCRs for each treatment. Amplification success rates (PCR+) and mean allele peak heights (APH) from the genotypic data were compared using paired *t* tests.

Adding BSA to DNA isolates was found to significantly increase PCR success by an average of around 20% (95% CI 12% - 28%; Fig. S3 (a), $p < 0.0005$). The addition of BSA to DNA isolates also significantly increased APH by an average of 1980 units (95% CI 1408-2252; Fig. S3 (b), $p < 0.0005$).

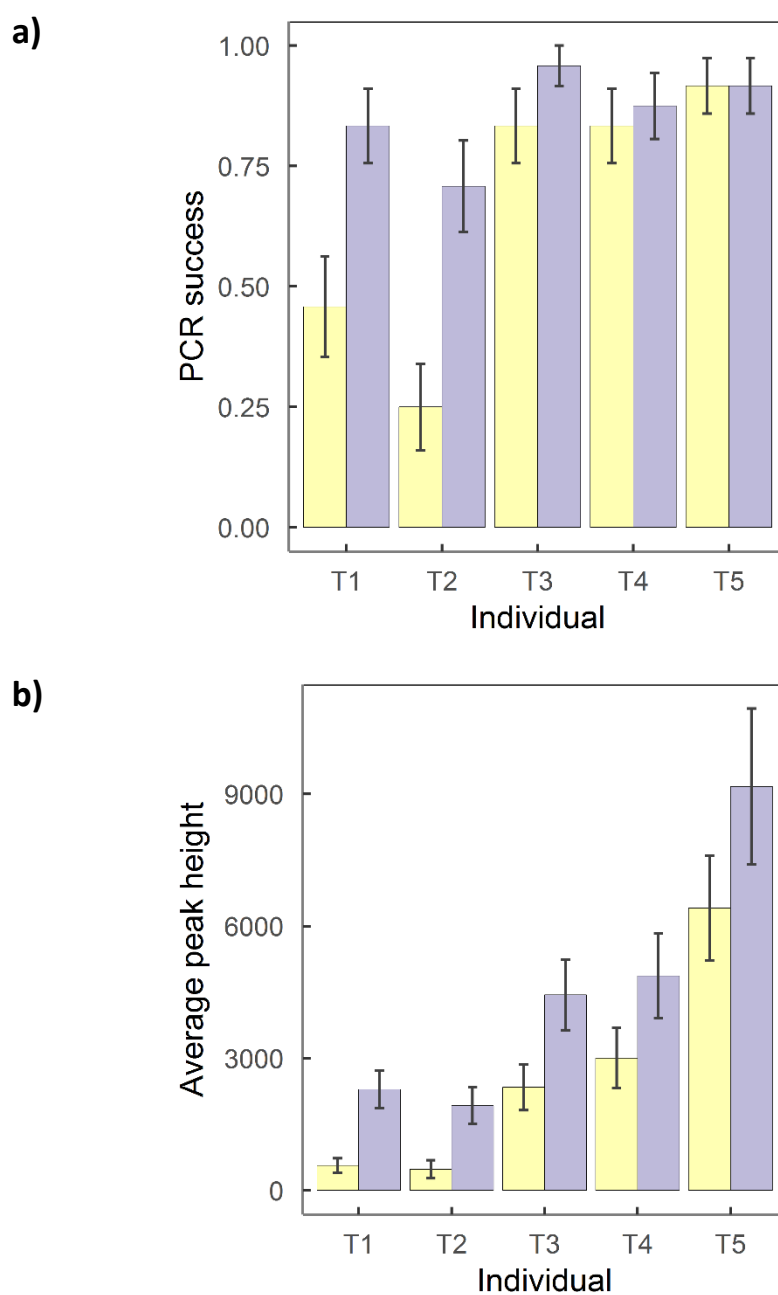


Figure S3 a) Amplification success and **b)** average peak height for each of the five DNA isolates (T1-T5) divided into two equal aliquots to which either nuclease free water or BSA (to a final concentration of 0.1 $\mu\text{g}/\mu\text{L}$) was added. The purple bars indicate samples with BSA added while the yellow bars represent samples with water added.

Chapter 6 | foreword

Together, chapters two to five demonstrate that koala DNA isolated from scats can be used to obtain reliable genotypic data. Genotypic data obtained from scats can then be used to study population differentiation, genetic diversity, gene flow and relatedness in koala populations, some of which are further described in chapters seven and eight.

In addition to koala DNA, DNA isolated from koala scats is also likely to contain dietary (eucalypt) and microbial DNA. To extend the applicability of DNA isolated from koala scats, chapter six aimed to demonstrate the detection of two pathogens affecting koala populations in DNA isolated from koala scats; the obligate intracellular bacterium, *Chlamydia pecorum* and the koala retrovirus (KoRV).

The work presented in chapter six shows that both *C. pecorum* and KoRV can be detected in DNA isolated from scats. DNA sequence data shows that *C. pecorum* strains detected in scats are identical or near identical to strains causing urogenital tract infections in koalas.

KoRV can be either endogenous or exogenous. Endogenous virus is transmitted vertically from parent to offspring. Because endogenous virus is integrated into the main chromosome, every cell will have at least one KoRV copy. Alternatively, KoRV may be transmitted horizontally between individuals; exogenous virus may be present in variable copy numbers in only some of the infected individual's cells. Chapter six suggests that both vertically and horizontally acquired infections are detected in DNA isolated from scats.

The ability to detect these pathogens in DNA isolated from koala scats has the potential to facilitate the collection of prevalence data across the koalas range, thereby providing a greater understanding of these pathogens, modes of transmission and their impact on koala populations. The prevalence of these infections in the South Gippsland koala population are explored in chapters nine and ten.



Chapter 6


A non-invasive tool for assessing pathogen prevalence in koala (*Phascolarctos cinereus*) populations: detection of *Chlamydia pecorum* and koala retrovirus (KoRV) DNA in genetic material sourced from scats



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A non-invasive tool for assessing pathogen prevalence in koala (*Phascolarctos cinereus*) populations: detection of *Chlamydia pecorum* and koala retrovirus (KoRV) DNA in genetic material sourced from scats

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Abstract Pathogenic diseases may threaten the viability of wild animal populations, especially when already vulnerable. The mitigation of risks associated with pathogenic infections in populations is an important factor in conservation strategies. Koalas are of conservation concern across the north of their range and are affected by two main pathogens; *Chlamydia pecorum* and the koala retrovirus (KoRV). This study tested whether DNA from *C. pecorum* and KoRV could be detected in genetic material isolated from koala scats. Detection of *C. pecorum* in scat isolated DNA samples was compared with results obtained from urogenital swabs collected from the same individuals as part of an independent study. The ability to detect KoRV in scats from both northern and southern regions of the koala's range was also assessed. There was a high level of concordance (5/6) between the detection of *C. pecorum* in DNA isolated from scats and urogenital swabs from the same individual. In positive samples, *C. pecorum ompA* genotypes were identical between DNA from scats and urogenital swabs in two out of three cases. In samples from the south of the koala's range, KoRV copy number was higher in DNA isolated from scats compared to DNA isolated from ear tissue, potentially indicating the detection of horizontally acquired infections. Our results demonstrate

the ability to detect *C. pecorum* and KoRV in DNA isolated from koala scats. This method will be useful for studying the prevalence, transmission and impact of these pathogens in wild populations which may subsequently inform conservation management strategies.

Keywords Non-invasive pathogen detection · Faecal samples · *Chlamydia pecorum* · Koala retrovirus (KoRV) · Wildlife health monitoring · Wildlife conservation · Quantitative PCR

Introduction

Pathogens infecting wild animal populations can affect the health of individuals and may lead to reduced fitness and mortality. Pathogenic diseases can reduce species viability and have been implicated in wild population declines (Tompkins et al. 2015). Consequently, pathogens may have a negative impact on biodiversity and their role as a potential driver of extinctions is becoming increasingly apparent (Smith et al. 2009). Greater data collection may facilitate characterisation and understanding of pathogen prevalence, transmission and impacts, and has the potential to inform and enhance management and conservation strategies.

Endemic to Australia, the koala (*Phascolarctos cinereus*) is an arboreal marsupial inhabiting the country's east coast, from Queensland to South Australia (Fig. 1), that has suffered dramatic population declines over the past two decades (Department of the Environment 2015). While the causes of decline are likely multifactorial, two pathogens have potential contributory roles: the obligate intracellular bacterium, *Chlamydia pecorum*, and the koala retrovirus (KoRV), both of which infect koalas throughout their

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Fig. 1 Koala distribution (shown by grey shading) across Australia adapted from Department of the Environment (2015)

range. *C. pecorum* commonly infects the eyes and/or urogenital tract of koalas (Markey et al. 2007). Ocular infection with *C. pecorum* may lead to severe keratoconjunctivitis (Cockram and Jackson 1981), while urogenital infections are known to result in cystitis and reproductive disease, which may lead to decreased fecundity or sterility (Martin and Handasyde 1990a; Obendorf and Handasyde 1990). A second species of *Chlamydia*, *C. pneumoniae*, also infects koalas but is usually associated with mild symptoms of disease (Jackson et al. 1999). There is evidence that different strains of *C. pecorum* have varying pathogenic potential (Mohamad et al. 2008, 2014) and that apparently asymptomatic chlamydial infections may have hidden impacts. In cattle, for example, reduced calf growth rates have been associated with asymptomatic *C. pecorum* infection (Poudel et al. 2012). The potential impacts of *C. pecorum* on individual koalas and koala populations highlight the need for better characterisation and understanding of the prevalence of *C. pecorum* infection.

KoRV is a retrovirus that is considered to be endogenous in northern koala populations and in the process of becoming endogenous in southern populations (Simmons et al. 2012). Although numerous strains of KoRV have been reported to date (Xu et al. 2013, 2015), KoRV-A is most commonly detected. Unlike most known endogenous viruses, proviral KoRV (viral DNA inserted into the host genome) is capable of producing active virus (Tarlinton et al. 2005); KoRV may therefore be transmitted vertically, from parent to offspring, or horizontally via contact transmission. Modes of KoRV horizontal transmission are not clear, however KoRVs closest known relative, the gibbon ape leukaemia virus (GALV) can also be transmitted both vertically, in the germline, and horizontally, via exposure to faeces or urine (Kawakami et al. 1977, 1978). Though not definitive, links have been made between KoRV infection and the incidence of lymphoma and leukaemia in koalas, as well as with clinical chlamydial infections (Tarlinton et al. 2005; Xu et al. 2013). In the north of the koala's range (from Port Macquarie northwards; Fig. 1), 100 % of koalas tested were found to be infected with KoRV. However, in the south-eastern state of Victoria, there is evidence that endogenous KoRV is less common, with virus integrated only into infected cells (exogenous infection) and a significant number of Victorian koalas tested are found to be KoRV negative (Simmons et al. 2012).

Knowledge of the epidemiology of *C. pecorum* and KoRV across the koala's range could identify pathogen free populations and/or populations at high risk of increased mortality due to infection. Detecting pathogenic organisms in wild animals is often challenging, as sampling typically involves animal capture which can be difficult, especially where the species is elusive or when population densities are low. Once located, site factors and an animal's position, often, in the case of koalas, in the tops of trees exceeding 30 metres in height within rugged terrain, may also limit accessibility and make capture difficult or impossible. Animal capture is usually followed by invasive procedures such as anaesthesia and the collection of urogenital or ocular swabs, tissue biopsies or blood extraction. These methods require veterinarians and/or specialist expertise, making widespread sampling costly and time consuming. Due to difficulties in sampling wild animals, the prevalence of pathogens is often determined using samples of convenience such as deceased individuals (e.g. roadkill) or those admitted to veterinary clinics or wildlife shelters. Though a subset of the wild population, the prevalence of infectious agents may be greater in the portion of the population that has met with misadventure or is unwell (entering shelters or clinics) compared to the overall wild population. Therefore, this type of sampling has the potential to lead to a biased result, which may not be

indicative of the true prevalence of the pathogen within the wider population.

As in other marsupials, both male and female koalas have a cloaca, a common external opening to the urogenital and gastrointestinal tracts (Archer et al. 1987). It therefore seems probable that dead *Chlamydia* infected cells and shed infectious particles (elementary bodies) may be detected in genetic material sourced from koala scats. The ability to carry out microsatellite genotyping using koala DNA isolated from scats (Wedrowicz et al. 2013) suggests that it should also be possible to detect KoRV in infected epithelial cells. In koalas originating from Queensland, the ability to detect endogenous KoRV (where all cells will contain at least one KoRV copy) in DNA isolated from koala scats has been demonstrated by Miyazawa et al. (2011). However, the ability to detect exogenous KoRV infections (in scat isolated DNA), where all somatic cells are not infected, is less certain. Given evidence that a large proportion of Victorian koalas may only carry horizontally acquired, exogenous KoRV infections (Simmons et al. 2012), this is important to determine.

The detection of koala pathogens in non-invasively sourced DNA could provide a viable alternative to invasive sampling. The ability to detect *C. pecorum* and KoRV in DNA isolated from koala scats would be a valuable tool with the potential to facilitate and expedite the collection of prevalence data for these infections across the koala's range. The objective of this study, therefore, is to determine whether *C. pecorum* and KoRV-A DNA can be detected in genetic material non-invasively sourced from koala scats, providing a tool that can assess the presence of these pathogens in wild koala populations.

Materials and methods

Chlamydia pecorum

Sample collection and DNA isolation

Scat samples were collected from six koalas in the Strzelecki Ranges, Victoria during March 2015. The six individual koalas from which scats were collected were also captured as part of an independent study carried out by the NSW Office of Environment and Heritage (Allen 2015). The purpose of the study carried out by Allen (2015) was to determine the prevalence of chlamydial infections in the Strzelecki Ranges koala population by capturing and collecting ocular and urogenital swabs from koalas. Results obtained from DNA isolated from urogenital swabs (six koalas: K01–K06) collected for the Allen (2015) study allowed direct comparison of *C. pecorum* presence detected by both methods. Scats located on the ground beneath

each koala captured by Allen (2015) were collected for the isolation of DNA according to the methods described by Wedrowicz et al. (2013). To assess detection reproducibility in DNA isolated from scats from the same individual, DNA was isolated from three different scats collected beneath each koala, resulting in three DNA isolates per individual. The concentration of total DNA isolated from scats was determined using the Qubit® 2.0 Fluorometer following the manufacturer's instructions.

Presence/absence assay for C. pecorum

Real time assays for *C. pecorum* targeting a 76 bp region of the *ompA* gene were carried out as per Pantchev et al. (2010). Reactions also incorporated TaqMan® Exogenous Internal Positive Control (IPC) Reagents in order to differentiate failed reactions, due to PCR inhibition, from true negative results. Amplification was carried out using the Applied Biosystems Step One Plus instrument using a presence absence protocol. PCR product using primers amplifying 1140 bp of the *ompA* gene (details below: *ompA* gene sequencing) was used to prepare 1:10 serial dilutions for standards ranging from 8×10^0 – 8×10^6 copies per reaction. Standards were used to assess reaction sensitivity including an IPC, as well as to estimate the number of chlamydial DNA copies in different samples. A standard PCR to determine the gender of the sampled individual was also carried out using DNA isolated from scats as outlined in Wedrowicz et al. (2013).

ompA gene sequencing

The presence of *C. pecorum* in samples which tested positive via real time PCR was confirmed by amplification of 1140 bp of the *ompA* gene. Amplification of *ompA* was carried out using BIO-X-ACT™ Short DNA Polymerase (Bioline). Reactions consisted of 1× OptiBuffer, 1× Hi-Spec additive, 2 mM MgCl₂, 0.5 mM each dNTP, 1 unit of BIO-X-ACT Short DNA Polymerase and 0.25 μM of each primer (*ompA*for and *ompA*rev; Kollipara et al. 2013) made up to 20 μL with water. DNA was initially denatured for 5 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min each and ending with a single final extension of 7 min at 72 °C. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequencing was carried out by the Australian Genome Research Facility (AGRF), Melbourne, Victoria. Resulting sequences were trimmed using Sequence Scanner V2 (Applied Biosystems) and nucleotide BLAST (NCBI 2016) used to determine the similarity of sequences to those previously reported. Genotypes were classified according to Kollipara et al. (2013), where *ompA* sequences with less than 1 % nucleotide differences to

previously reported sequences were designated by the same genotype letter followed by a prime symbol and a number to differentiate multiple variants with minimal base pair differences.

Comparison of scat and urogenital results

Results of *C. pecorum* presence in DNA isolated from scats were compared to *C. pecorum* presence in urogenital swabs obtained by Allen (2015). Results reported by Allen (2015) were determined independently by the Koala Health Hub (KHH) at The University of Sydney, New South Wales, Australia. DNA isolated from swabs testing positive for *C. pecorum* were provided for this study by the KHH allowing amplification and sequencing of the *ompA* gene to be carried out for swab isolated DNA as described above.

Koala retrovirus (KoRV)

Sample collection and DNA isolation

Koala scat samples were collected from wild individuals in the Strzelecki Ranges, South Gippsland ($n = 17$), Raymond Island ($n = 2$) and from koalas at the Koala Conservation Centre ($n = 6$) on Phillip Island, all in Victoria (total VIC samples; $n = 25$) (Fig. 1). Koala scats were also obtained from the Port Macquarie Koala Hospital in New South Wales (NSW, $n = 15$). Scats were collected and DNA isolated according to the methods outlined in Wedrowicz et al. (2013). To compare the results from scat samples with those from a commonly utilised invasive DNA source, DNA was also isolated from ear tissue samples ($n = 50$) from deceased individuals, collected by staff at the Southern Ash Wildlife Shelter (SAWS), Rawson, Victoria. Approximately 3 mm square was excised from ear samples and cut into small pieces. DNA was then isolated using the DNeasy® Blood & Tissue Kit (Qiagen) following the manufacturer's protocol.

KoRV-A PCR

Standard PCRs utilised KoRV-A specific primers published in Xu et al. (2013) alongside koala β -actin primers (Markey et al. 2007) to confirm the presence of koala DNA. Standard PCRs for KoRV-A were made up of 5 μ L GoTaq® Green Master Mix (Promega), 1.0 μ M of each KoRV-A primer, 0.1 μ g bovine serum albumin (BSA), 1 μ L of DNA template and water to give a final volume of 10 μ L. PCR cycling parameters were 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s followed by a final extension of 72 °C for 5 min. Standard PCR for koala β -actin consisted of 5 μ L GoTaq® Green Master Mix, 1.0 μ M of each KoRV-A

primer, 0.1 μ g BSA, 1 μ L of DNA template and water to bring the final volume to 10 μ L. PCR cycling parameters were 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and finished with a final extension of 72 °C for 5 min. To ensure the lower sensitivity of standard PCR assays was not resulting in an increased number of false negatives, real time PCR assays, using the same primers specific for KoRV-A (Xu et al. 2013), were carried out by the KHH on the same set of samples (VIC and NSW, $n = 40$) tested by standard PCR to independently validate results. Real time PCRs were carried out on both undiluted and 1:10 dilutions of DNA isolates.

KoRV-A copy number

DNA from tissues ($n = 50$) and additional scat samples ($n = 117$) collected in the South Gippsland region were also screened for KoRV (data not shown). Using a subset of randomly selected positive samples, KoRV copy number was compared between Victorian scat samples (VIC, $n = 13$) and ear tissue samples (TISS, $n = 13$) along with scat samples collected from New South Wales (NSW, $n = 8$). This was carried out using real time PCR with KoRV-A primers (Xu et al. 2013) and β -actin primers (Markey et al. 2007) to standardise copy number. Standards were produced for the KoRV-A and β -actin assays by amplifying the two targets using standard PCR. KoRV-A and β -actin PCR product was then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Serial 1:10 dilutions ranging approximately from 10^0 to 10^6 copies of KoRV-A or β -actin PCR product were then used as standards for the estimation of copy number. Both PCRs were carried out using 10 μ L of SYBR® Green Master Mix (Applied Biosystems), 0.25 μ M of each primer and 2 μ L of DNA template made up to a total of 20 μ L with water and cycling parameters consisting of 50 °C for 2 min (UDG activation), 95 °C for 2 min (polymerase activation) followed by 50 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt curve was produced to check for non-specific products. All standards, controls and samples were tested in duplicate.

KoRV-A sequencing

A subset of PCRs positive for KoRV ($n = 6$) were sequenced to confirm the detection of KoRV-A. Primers were designed using published KoRV-A sequence (Genbank AF151794.2; Hanger et al. 2000) and Primer-BLAST (Ye et al. 2012) to target a 1115 bp region of the KoRV *env* gene; primers were designated KoRV-*env*1-F (5'-AGACG GGAAGTGTCGTTTGG-3') and KoRV-*env*1-R (5'-GGG GGTGAGGCCAGAATTAC-3'). Positive samples were

amplified using 10 µL GoTaq® Green Master Mix, 0.5 µM of each primer and 0.1 µg BSA made up to a final volume of 20 µL. PCR products were purified using the ISOLATE PCR and Gel Kit (Bioline) and sequencing was carried out by AGRF.

Results

Chlamydia pecorum

Assay sensitivity and reproducibility

The detection limit for the *C. pecorum* assay, with the addition of an internal positive control, was consistent with the limits reported by Pantchev et al. (2010) at eight copies of chlamydial DNA per PCR (Online resource 1, Fig. S1). *C. pecorum* DNA was detected in all three scats from each positive individual indicating that, when present, *C. pecorum* is consistently found in DNA isolated from koala scats. The total number of *C. pecorum* copies detected in DNA isolated from urogenital swabs was much higher compared to scats (Table 1). Results obtained from scat or

urogenital swab isolated DNA were generally concordant (Table 2), but, one of the six scat DNA isolates was positive for *C. pecorum* while the urogenital swab was negative (K06).

C. pecorum copy number and DNA concentration

DNA isolated from scats ranged in concentration from 1.4 ng/µL up to 42 ng/µL (Table 1; Fig. 2). The estimated copy number of *C. pecorum* increased with increasing DNA concentration (Fig. 2). Different scats from the same individual were consistently either positive or negative, though there was variation in the number of *C. pecorum* copies detected for independent scats from the same individual (Table 1). Of the fifteen DNA samples isolated from scats positive for *C. pecorum*, twelve had less than 10 *C. pecorum* copies per ng of DNA isolated while the remaining three samples had somewhat higher copy numbers; 15, 37 and 74 *C. pecorum* copies per ng of DNA (Table 1). Compared to DNA isolated from scats for each individual, *C. pecorum* copy number was on average, three (K02), ten (K01 and K04), and 3000 (K05) times greater in DNA isolated from swabs.

Table 1 *C. pecorum* copy number and DNA concentration for each isolate tested in this study

Koala ID	Sample	Copy number	DNA concentration (ng/µL)	Copy number per ng of total DNA
K01	Scat 1	1.71	1.42	1.21
	Scat 2	8.86	1.91	4.63
	Scat 3	14.4	1.60	9.00
	Swab	84.8	nt	nt
K02	Scat 1	155	2.10	73.6
	Scat 2	21.9	3.92	5.57
	Scat 3	8.57	2.54	3.38
	Swab	203	nt	nt
K03	Scat 1	NA	14.0	NA
	Scat 2	NA	7.88	NA
	Scat 3	NA	6.46	NA
	Swab	Negative	nt	NA
K04	Scat 1	624	41.6	15.0
	Scat 2	241	6.52	37.0
	Scat 3	358	42.2	8.49
	Swab	3840	nt	nt
K05	Scat 1	16.7	6.62	2.53
	Scat 2	34.2	9.10	3.76
	Scat 3	23.4	10.9	2.16
	Swab	74,800	nt	nt
K06	Scat 1	12.9	1.90	6.81
	Scat 2	8.91	5.46	1.63
	Scat 3	7.21	4.38	1.65
	Swab	Negative	nt	NA

NA not applicable, nt not tested

Table 2 Summary of results for PCRs targeting *C. pecorum* in DNA isolated from koala scats with comparison to results reported by Allen (2015) from swab samples

Koala ID (Allen 2015 ID)	DNA isolated from scats (this study)					DNA isolated from urogenital swabs (Allen 2015)		
	XY PCR	Real time assay	<i>C. pecorum</i> copy number per ng (mean \pm SE)	<i>ompA</i> genotype	Sequence similarities	Gender	Real time assay	<i>ompA</i> genotype
K01 (STRZ009)	Female	Positive	4.95 \pm 2.25	F'3	99 % identity to KU214246.1	Female	Positive	F'3 (99 % identity to KF150135.1)
K02 (STRZ010)	Female	Positive	27.5 \pm 23.0	F'3	99 % identity to KU214246.1	Female	Positive	M (100 % identity to KU214247.1)
K03 (STRZ011)	Female	Negative	NA	Negative	NA	Female	Inconclusive ^b	NA
K04 (STRZ012)	Female	Positive	20.1 \pm 8.60	C'1	99 % identity to KU214245.1	Female	Positive	C'1 (99 % identity to KU214245.1)
K05 (STRZ013)	Male	Positive	2.82 \pm 0.485	–	Sequence unreadable ^a	Male	Positive	F (100 % identity to KF150135.1)
K06 (STRZ014)	Female	Positive	3.36 \pm 1.72	F'3	99 % identity to KU214246.1	Female	Negative ^c	NA

ompA genotypes and comparisons are based on those reported by Kollipara et al. (2013) and Legione et al. (2016)

NA not applicable, SE standard error of the mean

^a Unreadable sequence obtained, possibly due to the presence of multiple strains

^b Sample was positive for the *C. pecorum* specific assay (but with low copy number, $C_T = 38$), but negative using the *Chlamydia* genus and *C. pneumoniae* assays (Allen 2015)

^c Sample was negative using species specific (both *C. pecorum* and *C. pneumoniae*) assays and positive (but with low copy number, $C_T = 37$) using a *Chlamydia* genus PCR (Allen 2015)

ompA genotypes detected using DNA isolated from scats and swabs

Of the five *Chlamydia* positive samples detected using DNA isolated from scats, four were successfully sequenced at *ompA* with two different genotypes being detected (Table 2). Referring to the types reported by Kollipara et al. (2013), genotypes were most closely related to *ompA* types F (K01, K02 and K06; F'3, $n = 3$, two bases difference to genotype F, both non-synonymous) and C (K04; C'1, $n = 1$, one base difference, synonymous).

Each of the four positive urogenital swab samples produced different *ompA* sequences to one another (Table 2). These included F'3 (K01) and C'1 (K04), both of which matched the sequences obtained from counterpart scat samples. An *ompA* sequence 100 % identical to genotype F described by Kollipara et al. (2013) was detected in the swab sample of koala K05, but the *ompA* sequence obtained for K05 using scat isolated DNA could not be read, possibly due to the presence of multiple strains. The remaining swab sample (K02) was identified as genotype M (Legione et al. 2016) and differed to the corresponding *ompA* sequence from the paired scat isolated DNA (F'3). The *ompA* sequence (genotype M) obtained from swab DNA for individual K02 was noted to have some level

background sequence while all other sequences were completely free of any background noise.

Koala retrovirus (KoRV)

Comparison between standard and real time PCR results

The DNA concentration isolated from the 40 scat samples tested for KoRV presence ranged from undetectable (<0.10 ng/ μ L, $n = 3$ NSW samples) to 41 ng/ μ L with a mean of 4.2 ng/ μ L and median of 1.2 ng/ μ L (Online resource 1: Table S2). Standard PCR results (undiluted template) were identical to the real time PCR results (undiluted template), except for three (12.5 %) South Gippsland positives which tested negative by real time PCR when isolates were diluted 1:10 (Online resource 1: Table S2). KoRV-A was detected in 24 of the 40 samples tested (60 %). Using both standard and real time PCR all six Phillip Island samples tested in this study were negative for KoRV while all 15 NSW samples tested positive. As expected, β -actin and KoRV-A cycle thresholds decreased with increasing amounts of total DNA concentration obtained from koala scats (Fig. 3), indicating greater amounts of koala and KoRV DNA present in DNA isolates with higher DNA concentration.

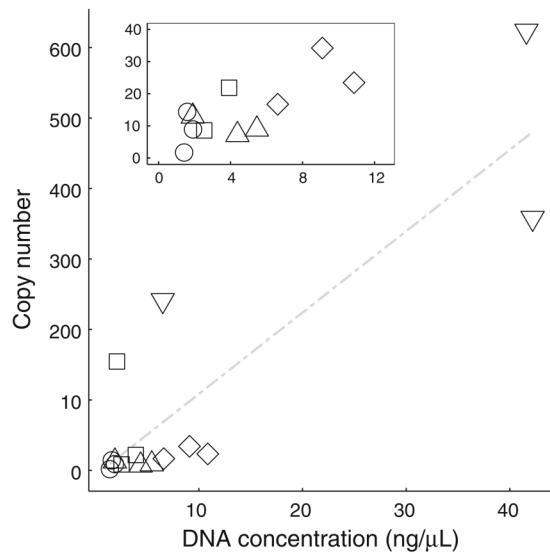


Fig. 2 Plot showing the relationship between *C. pecorum* copy number and DNA concentration in DNA isolated from scat samples testing positive for *C. pecorum* (K01, K02, K04, K05 and K06). Symbols represent the independent scats from the same individual: K01 (open circle), K02 (open square), K04 (open inverted triangle), K05 (open diamond) and K06 (open triangle). The overall regression equation was *C. pecorum* copy number = $-7.0 + 11.6 \times \text{DNA concentration (ng/}\mu\text{L)}$ ($R^2 = 74.3$, $p < 0.0005$). The cluster of data points with DNA concentration below 12.5 ng/μL and copy number below 40 *C. pecorum* copies per genome is shown in greater detail in the inset at the top left of the figure

Confirmation of KoRV-A presence by sequencing

A 1115 bp segment of the KoRV *env* gene was sequenced for a subset of positive samples to confirm the detection of KoRV DNA. Sequences of samples from NSW koalas ($n = 2$) were 100 % identical to KoRV-A sequence (AF151794.2; Hanger et al. 2000). All samples from Victorian koalas that were sequenced ($n = 4$) had six identical nucleotide base changes in the *env* segment. The six polymorphisms in the Victorian samples were all synonymous, not having an effect on the amino acid sequence of the *env* gene.

Differences in KoRV-A copy number between sample groups

Quantitative PCR revealed significant differences in copy number between scat samples from NSW and Victoria. On average, more than four KoRV-A copies per genomic unit were detected in scats sampled in NSW, whereas less than one copy per genomic unit was detected in Victorian scat samples (Fig. 3; $p = 0.01$). This is concordant with results reported by Simmons et al. (2012) further supporting the hypothesis that most infections in Victoria are exogenous, as endogenous proviral DNA would be expected to be

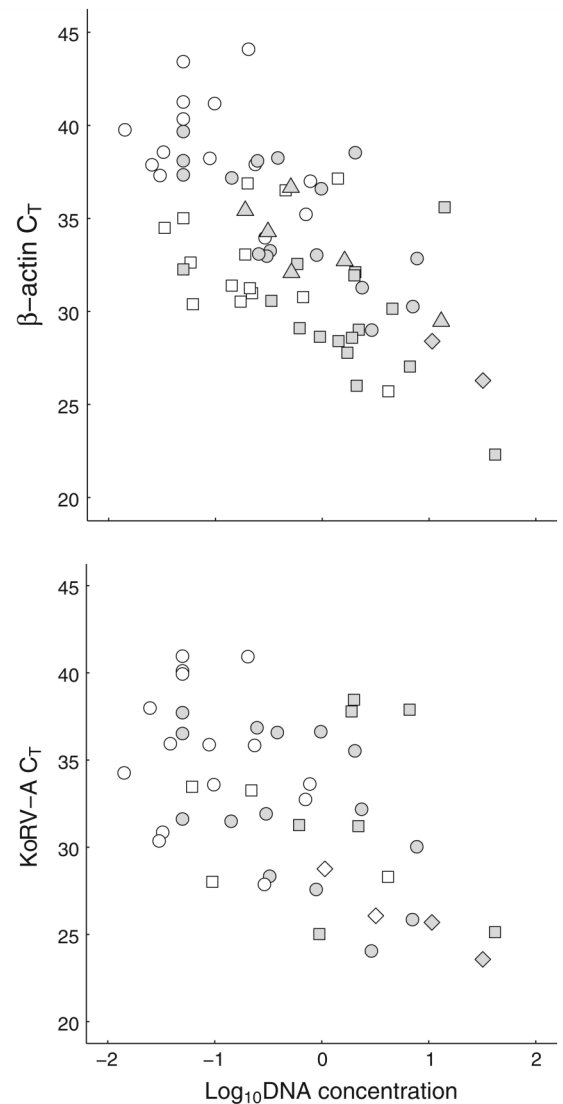


Fig. 3 The relationship between cycle threshold (C_T) and DNA concentration for positive β -actin and KoRV-A real time PCRs. Symbols indicate samples from NSW (open circle), South Gippsland (open square), Raymond Island (open diamond) and Phillip Island (open triangle). Unshaded symbols denote DNA isolates that were diluted 1:10 while shaded symbols indicate undiluted samples. The regression fit for the β -actin PCR is $C_T = 32.5 - 3.7 \log_{10}\text{DNA}$ ($R^2 = 42\%$, $p = 1.1 \times 10^{-9}$) while the KoRV-A PCR had a regression fit of $C_T = 31.6 - 2.9 \log_{10}\text{DNA}$ ($R^2 = 26\%$, $p = 1.9 \times 10^{-4}$)

present in every cell if acquired vertically via the germline. Simmons et al. (2012) showed that KoRV is endogenous in northern koala populations, and is inserted into the genome multiple times, with an average of 165 KoRV copies per cell reported for Queensland individuals; the mean of four KoRV copies per genomic unit value reported for NSW koalas in this study is substantially lower. KoRV-A copy number also differed significantly between scat and ear tissue samples collected in South Gippsland, with scats having on average, 30 times as many KoRV-A copies per

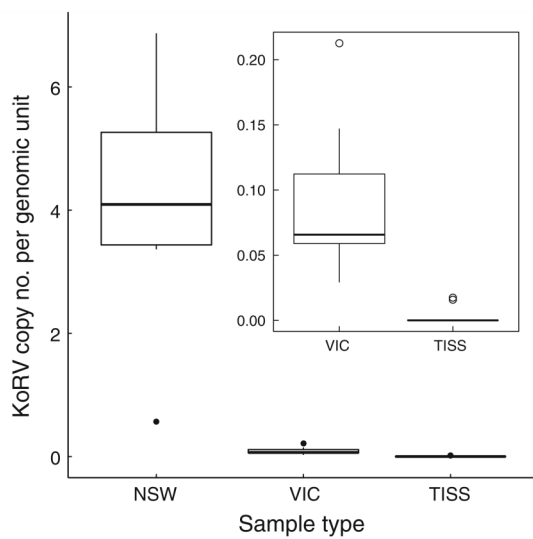


Fig. 4 Differences in KoRV-A copy number per genomic unit (β -actin) between sample groups. Scat samples from New South Wales (NSW, $n = 8$) had a mean of 4.15 ± 0.66 KoRV-A copies per genomic unit, scat samples from Victoria (VIC, $n = 13$) had a mean of 0.087 ± 0.014 copies per genomic unit and tissue samples from Victoria (TISS, $n = 13$) had 0.003 ± 0.002 KoRV-A copies per genomic unit. All groups differed significantly from one another ($p \leq 0.0005$). The inset shows the data for Victorian scat (VIC, $n = 13$) and tissue samples (TISS, $n = 13$) on a smaller scale

genomic unit than tissue samples (Fig. 4; $p < 0.0005$). KoRV-A in DNA isolated from ear tissue averaged 0.003 ± 0.002 copies per genomic unit while DNA isolated from scats had a mean of 0.09 ± 0.01 KoRV-A copies per genomic unit.

Discussion

Pathogenic organisms can impact wild animal populations by reducing animal health, longevity and the ability to successfully reproduce. Impacts such as these may result in decreased population size causing losses in genetic diversity (Frankham et al. 2012) which, together with other threatening processes such as habitat loss and fragmentation, may greatly increase the risk of inbreeding, deleterious genes and extirpation. Given declines in koala numbers and the 2012 listing of northern koala populations as vulnerable under the *Environmental Protection and Biodiversity Conservation Act 1999* (Department of the Environment 2015) it is imperative that the prevalence of *C. pecorum* and KoRV within the koala population is determined. Obtaining such data via swabs however, can be costly and time consuming, especially for species such as the koala, which are difficult to capture. DNA isolated from a single scat can provide information on the presence and characteristics of *C. pecorum* and KoRV. Both *C.*

pecorum and KoRV were detected in DNA isolated from koala scats. The non-invasive collection of samples therefore has potential to greatly increase sample size in studies of koala pathogens, without the requirements of capturing, handling or even observing an animal. This allows for the collection of more samples from wider and more diverse geographic areas, increasing both the spatial distribution and number of samples obtained for studies of pathogens in wildlife. Such data is critical for effective species management, especially in the case of inter-population translocations or releases proposed by wildlife managers or carers.

In Australia, the translocation of wild animals is common, with 54 species ($\sim 14\%$) of Australian mammals having at least one documented translocation (Short 2010). Additional undocumented movements of animals carried out by wildlife carers releasing individuals after rehabilitation are also commonplace in Australia (Guy and Banks 2012; Reid 2014). Translocations are often carried out for conservation reasons, however the risk of transferring pathogens between endemic and introduced individuals is an important consideration that requires prior assessment (Leighton 2002) and post translocation surveillance (Mathews et al. 2006). Severe *Chlamydia* infections are more likely in individual koalas not previously exposed to these bacterial infections (Martin and Handasyde 1990b). The inadvertent introduction of *C. pecorum* to populations that have historically been free of infection, therefore, has the potential to result in devastating consequences. The effects of exposure to novel strains are also important considerations due to possible differences in strain pathogenicity (Mohamad et al. 2008, 2014). Although the pathogenic effects of KoRV are not entirely clear at present, preventing transmission of the virus to KoRV free populations is important. Obtaining baseline data on KoRV prevalence in wild populations will enable monitoring of infection rates and future changes that may occur.

C. pecorum has also been found to infect other Australian marsupials such as the greater glider (*Petauroides volans*), the short-eared brushtail possum (*Trichosurus caninus*) and the western barred bandicoot (*Perameles bougainville*) (Bodetti et al. 2003). Other than the koala, limited research into the effects of this pathogen on marsupial populations has been carried out. Given that urogenital infections with *Chlamydia* may be subclinical, yet affect reproduction, the presence of this bacteria in marsupial populations may potentially be an under recognised cause of population declines. The use of scat sourced DNA could provide a simple and useful method for screening and characterising *Chlamydia* infections in a range of marsupial species. Similarly, a retrovirus similar to KoRV and GALV has been detected in an Australian rodent, *Melomys burtoni* (Simmons et al. 2014). Using DNA

sourced non-invasively from scats, sampling power and the ability to identify other potential retroviral hosts and/or cross species transmissions may be greatly increased. This method could therefore also be of value to the study of these bacterial and viral pathogens in other Australian marsupials. Screening for potential pathogens, utilising non-invasive genetic sampling techniques as described here, may be a useful addition to studies of other mammals.

This study demonstrates that *C. pecorum* strains, also found to cause urogenital infection in koalas (Kollipara et al. 2013; Legione et al. 2016), can be detected in DNA isolated from koala scats. It is important to recognise that infection with *C. pecorum* does not mean that an individual will have symptoms of chlamydiosis. The presence of *C. pecorum* does, however, provide an indication that the individual carries the pathogen and is probably capable of transmitting the bacteria to other individuals. Conversely, a negative result does not necessarily show that the individual is free of infection, since latent infections or low level shedding of infectious particles could result in too few *C. pecorum* particles present on koala scats for detection.

Results of the *C. pecorum* assay were largely concordant between scat and swab samples, although some differences were also noted. One individual (K06) tested positive for *C. pecorum* in DNA isolated from scats and negative using DNA isolated from the urogenital swab. This may indicate that insufficient biological material was obtained during swabbing or that infected cells detected in scat DNA were located at other reported sites of chlamydial infection such as the gastrointestinal tract (Burach et al. 2014). Another explanation is that scats were from another koala having visited the same tree rather than the koala captured; genotyping of both the scat and swab DNA isolates would be required to confirm or exclude this possibility. The *ompA* genotype obtained for one individual (K02) differed between scat and swab samples; the scat sample providing the F'3 genotype and the swab sample the M genotype. The M genotype sequence obtained from this swab was however the only sequence with a substantial amount of background, potentially indicating the presence of multiple strains or alternatively, sample contamination. Further investigation is needed to determine whether differences in the *ompA* type detected using different sample types from the same individual is due to the collection of scats from unintended individuals, as described above, or to multiple strain infections, potentially located at different anatomical sites (Burach et al. 2014). For example, infections of the gastrointestinal tract may be present in scat samples, but absent from urogenital swab samples.

Compared to DNA isolated from swabs, the number of *C. pecorum* copies detected in scat DNA was much lower and the number of estimated *C. pecorum* copies varied

between DNA samples isolated from independent scats from the same individual. Although positive samples were consistently positive across the three separate scats, it is possible that the bacteria may not be detected when the rate of shedding of infectious particles is low. Copy number variability was at least, in part, related to the concentration of DNA obtained from scats. Further work to determine the minimum quantity of total DNA needed for reliable detection of *C. pecorum* in DNA isolated from scats, from individuals with various infectious loads, would be appropriate. Testing multiple independent scats from the same individual may increase confidence in results obtained. The only male koala included in this study (individual K05) was estimated to have a *C. pecorum* copy number that was between 2200 and 4500 times lower in DNA sourced from scats compared to DNA sourced from swabs, while the females in the study had copy numbers in scats that were between one and fifty times lower. Differences in the anatomy of female and male koalas could potentially affect the ability to detect *C. pecorum* in males and female scats; this requires further investigation.

Compared to Victorian tissue samples, significantly greater amounts of KoRV-A were detected in DNA isolated from Victorian scat samples (Fig. 4). This difference suggests the possible detection of infection acquired horizontally (exogenous infection) in Victorian koalas. All northern NSW samples in this study tested positive for KoRV, while no samples were KoRV positive in the Phillip Island group of samples. This is consistent with results previously reported by Simmons et al. (2012), who found all Phillip Island (Victoria) samples tested to be KoRV negative and all samples tested in the Port Macquarie (NSW) area to be KoRV positive. Apart from one sample, standard and real time PCR assays of the samples tested here produced equivalent results. Where presence or absence of KoRV is all that is required for a study, standard PCR can also provide useful data. The small number of diluted positive samples that tested negative by real time PCR indicates that KoRV may not be detected when DNA concentration is quite low. One way to address this may be to screen DNA samples for quantity and quality in order to ascertain the suitability of samples for testing. Additionally, if KoRV detected in scat isolated DNA originates from exogenous infections, the number of cells in which KoRV DNA has integrated may be highly variable, resulting in low copy numbers in some samples. Consequently, until further information is obtained, presence only studies are likely to be most suitable using these methods. In Victorian samples, higher KoRV-A copy number was found in DNA isolated from scats compared to ear tissue, suggesting that koala scats provide a practical source of DNA for detecting the presence of KoRV infections acquired both vertically and horizontally.

Koala conservation research will benefit from the use of an integrated approach, employing the relatively simple method for detecting *C. pecorum* and KoRV described here. Genetic material isolated from koala scats has been shown to provide DNA that can be reliably used to obtain genotypic data, DNA sequence information and to identify gender (Wedrowicz et al. 2013). DNA isolated from scats can provide information on population substructure, relatedness between individuals, gene flow, migration and hybridisation using genotyping (Czarnomska et al. 2013; Piggott et al. 2006; Stenglein et al. 2011). The gender of the individual sampled can be determined by PCR (Wedrowicz et al. 2013), while sequencing of mitochondrial DNA can provide phylogenetic information suitable for evolutionary analyses (Houlden et al. 1999). Genotypic data can also be paired with habitat and environmental models to look for factors that may impede or facilitate gene flow across the landscape using landscape genetics (Storfer et al. 2006).

The ability to detect pathogens in DNA isolated from koala scats may supply critical information that can be combined with genetic data to provide a multidimensional picture of koala biology. Since pathogen DNA sequences can be obtained using this method, non-invasive sampling may also permit studies of pathogen diversity, transmission and spread throughout wild populations. In the case of *C. pecorum*, the likelihood of particular *C. pecorum* strains having high or low virulence could also be investigated using *ompA* markers along with additional *C. pecorum* DNA markers such as *incA* and ORF663 (Mohamad et al. 2014). A general trend of increased *C. pecorum* shedding has been shown for individuals with increasing severity of chlamydial disease (Wan et al. 2011) and there is also evidence of differing infectious load between *C. pecorum* strains (Legione et al. 2016). It may, therefore, also be useful to differentiate individuals with high or low loads of *C. pecorum* using DNA isolated from scats. However, further comparisons of copy number between swabs and scats would be required in order to more accurately gauge the variability of *C. pecorum* copy number in scat isolated DNA and the number of scats needed to give a reliable estimate of infectious load. Pathogen detection from scats could be paired with ecological studies, thereby revealing whether infection might have an effect on behaviour or movement. This information has the potential to provide increased knowledge of many aspects of *C. pecorum* and KoRV infection in koala populations including transmission, pathogenicity and host effects. Together, the plethora of information that is able to be gained from a single koala scat can be used to promote a holistic approach to koala conservation and management.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval A research permit was obtained under provisions of the Wildlife Act 1975 and National Parks Acts 1975 from the Department of Sustainability and Environment (Permit No. 10004020). Research was also approved by the Monash University Biological Sciences Animal Ethics Committee (AEC No. GIPP/2011/03).

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Chapter 6 | Supporting information

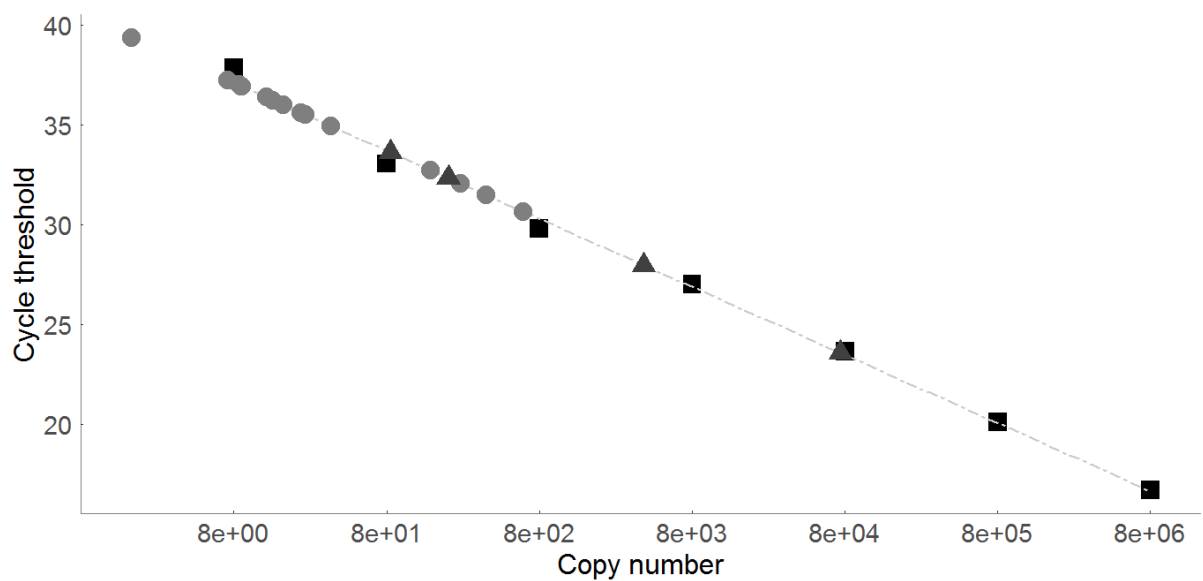


Figure S1 Summary of the *C. pecorum* real time assay results. Standard curve of the *Chlamydia pecorum* assay used to calculate copy number for *C. pecorum* found in DNA isolated from scats or urogenital swabs. Standards were produced using serial dilutions of *C. pecorum ompA* PCR product between 8 and 8×10^6 copies of *ompA* DNA (■). The equation of the regression line for the standards was $C_T = -3.4\log_{10}(\text{copy number}) + 40.2$ with an r^2 of 0.996 and reaction efficiency of 97%. The results for all scat samples (●) and swab samples (▲) are also shown.

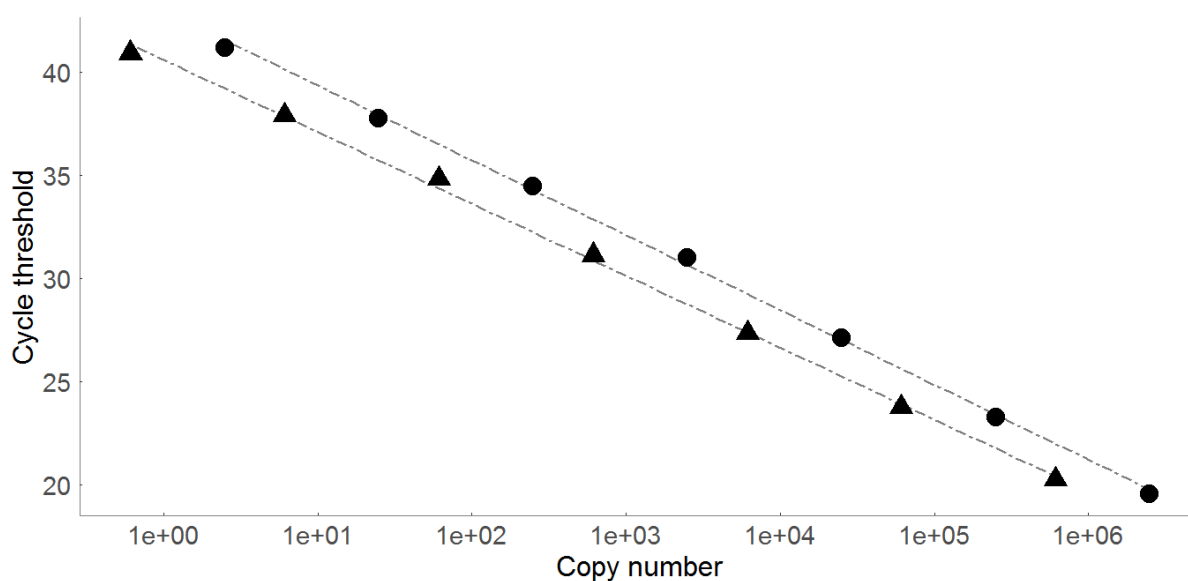


Figure S2 Real time PCR KoRV-A assay standard curves. Standard curve for the β -actin (●) and KoRV-A (▲) PCR assays used to estimate copy number. The equation of the β -actin regression line was $C_T = -3.6\log_{10}(\text{copy number}) + 43.0$ with an r^2 of 0.999. Reaction efficiency was 89%. The equation of the KoRV-A regression line was $C_T = -3.5\log_{10}(\text{copy number}) + 40.6$, r^2 was 0.998 and reaction efficiency was 94%.

Table S1 The number of *C. pecorum* copies detected in DNA isolated from scats. The four urogenital swabs were calculated to have 85 (K01), 203 (K02), 3842 (K04) and 74765 (K05) *Chlamydia* DNA copies.

DNA source	<i>n</i>	<i>C. pecorum</i> copy number		
		Minimum	Mean \pm SE	Maximum
Scats	15	2	102 \pm 46	622
Swabs	4	85	19724 \pm 18368	74765

Table S2 Individual results for KoRV-A and β -actin assays by standard PCR and real-time PCR (conducted by the KHH). Reaction results are categorised as positive (+) or negative (-). Samples that were not tested are denoted by “nt”. Sample regions included Raymond Island (RI), Phillip Island (PI), the Strzelecki Ranges in South Gippsland (STZ) and New South Wales (NSW). Samples were tested using standard PCR and undiluted DNA isolate, real-time PCR and undiluted DNA isolate and real-time PCR with DNA isolate diluted 1:10. Results that were not identical between all tests are indicated by an asterisk.

Sample details			KoRV-A PCRs			beta-actin PCRs		
Sample ID	Region	DNA concentration (ng/ μ L)	Standard PCR	Real-time PCR	Real-time PCR (1:10 dilution)	Standard PCR	Real-time PCR	Real-time PCR (1:10 dilution)
TS-01	RI	32	+	+	+	+	+	nt
TS-02		11	+	+	+	+	+	nt
TS-03	PI	0.19	-	-	-	+	+	nt
TS-04		0.31	-	-	-	+	+	nt
TS-05		1.6	-	-	-	+	+	nt
TS-06		0.51	-	-	-	+	+	nt
TS-07		0.51	-	-	-	+	+	nt
TS-08		13	-	-	-	+	+	nt
TS-09	STZ	0.95	+	+	+	+	+	nt
TS-10		2.0	-	-	-	+	+	nt
TS-11		2.2	+	+	+	+	+	+
TS-13		0.58	-	-	-	+	+	+
TS-14		1.7	-	-	-	+	+	+
TS-15		< 0.1	-	-	-	+	+	+
TS-17		4.5	-	-	-	+	+	+
TS-18		1.4	-	-	-	+	+	+
TS-19		2.1	-	-	-	+	+	+
TS-20		14	-	-	-	+	+	+

Table S2 Individual results for KoRV-A and β -actin assays by standard PCR and real-time PCR (*continued*).

Sample details			KoRV-A PCRs			beta-actin PCRs		
Sample ID	Region	DNA concentration (ng/ μ L)	Standard	Real-time PCR	Real-time PCR (1:10 dilution)	Standard	Real-time PCR	Real-time PCR (1:10 dilution)
TS-21	STZ	42	+	+	+	+	+	+
TS-16		0.62	+	+	+	+	+	+
TS-22		6.6	+	+	- *	+	+	+
TS-23		1.9	+	+	- *	+	+	+
TS-24		2.0	- *	+	- *	+	+	+
TS-32		0.25	-	-	-	+	+	+
TS-25	NSW	2.4	+	+	+	+	+	+
TS-26		0.89	+	+	+	+	+	+
TS-27		0.25	+	+	+	+	+	- *
TS-28		0.38	+	+	+	+	+	- *
TS-29		0.33	+	+	+	+	+	+
TS-30		0.3	+	+	+	+	+	+
TS-31		0.14	+	+	+	+	+	+
TS-33		7.0	+	+	+	+	+	+
TS-34		2.1	+	+	+	+	+	+
TS-35		7.7	+	+	+	+	+	+
TS-36		< 0.1	+	+	+	+	+	+
TS-37		< 0.1	+	+	+	+	+	+
TS-38		< 0.1	+	+	+	+	+	+
TS-39		0.98	+	+	+	+	+	+
TS-40		2.9	+	+	+	+	+	+

Table S3 Summary of samples tested for KoRV-A using quantitative PCR. Data is presented in Fig. 4 of the manuscript.

Region	Sample type	KoRV-A copies per genomic unit
New South Wales	Scat	5.7 E-01
	Scat	5.6 E+00
	Scat	6.9 E+00
	Scat	3.4 E+00
	Scat	3.5 E+00
	Scat	4.4 E+00
	Scat	3.8 E+00
	Scat	5.2 E+00
Victoria	Tissue	3.4 E-05
	Tissue	2.1 E-05
	Tissue	1.0 E-06
	Tissue	4.4 E-06
	Tissue	6.0 E-05
	Tissue	9.3 E-06
	Tissue	2.7 E-05
	Tissue	1.6 E-02
	Tissue	3.6 E-06
	Tissue	2.1 E-06
	Tissue	8.6 E-06
	Tissue	1.0 E-05
	Tissue	1.7 E-02
	Scat	5.9 E-02
	Scat	1.1 E-01
	Scat	1.0 E-01
	Scat	2.1 E-01
	Scat	1.5 E-01
	Scat	1.1 E-01
	Scat	4.1 E-02
	Scat	5.2 E-02
	Scat	6.3 E-02
	Scat	6.6 E-02
	Scat	2.9 E-02
	Scat	6.6 E-02
	Scat	7.0 E-02



Part 2

Investigating the South Gippsland koala population using molecular methods





Chapter 7 | foreword

Genetic structure and diversity within populations is the result of pressures having occurred across time, both in the remote and recent past. Patterns of genetic structure, diversity and gene flow may have been affected by rapid changes to landscapes and habitat, and increased human interference with wildlife occurring after European settlement in Australia (post 1788). Knowledge of impacts on koala populations in South Gippsland occurring over the past 200 years or so are therefore likely to be helpful when interpreting current patterns of genetic structure and diversity. The following chapter therefore provides a natural history of koalas and landscape changes in the South Gippsland region of Victoria. It is intended to summarise past impacts that may have played a role in shaping the genetic structure of the contemporary koala population in South Gippsland.



Chapter 7

Landscape, koalas and people: A historical account of koala populations and their environment in South Gippsland



Photo source: State Library of Victoria, Cyril Grant Lane, ca. 1900 - ca. 1912, Author, skinning a koala (taken with a self-acting shutter), URL: <http://handle.slv.vic.gov.au/10381/52767>

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Landscape, koalas and people: A historical account of koala populations and their environment in South Gippsland

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ABSTRACT

We present an ecological history of the koala (*Phascolarctos cinereus*) population and its environment in South Gippsland, Victoria, both pre- and post- European settlement. We consider the role that the region's history may have had on the genetic structure of the current koala population in South Gippsland, which is the only known koala population in Victoria that does not originate from animals re-introduced as part of the Victorian translocation program.

Following European colonisation of Australia, a range of anthropogenic factors, including hunting for the fur trade, resulted in widespread population declines for the koala. In Victoria, the situation was extreme. Currently, many koala populations in Victoria are derived from only a few individuals which existed less than 120 years ago. These populations therefore have comparatively low genetic diversity, a factor that plays a key role in long term population viability.

In Victoria, the koala is not listed as a threatened species. Despite the low genetic diversity of most populations, the species is widely distributed across the state, and relatively common. Indeed, some populations are considered overabundant. However, many koala populations are not abundant, and population data are lacking for most. The South Gippsland koala population is of high conservation significance as it has greater genetic diversity compared to other Victorian populations, though there is little additional data to inform its conservation.

An improved understanding of genetic diversity and gene flow between populations across the koala's range is required to guide the conservation of genetic diversity in this species. Monitoring population size, health and genetic relationships both within and between koala populations will enable better conservation outcomes.

Key words: *Phascolarctos cinereus*, Aboriginal history, translocation, *Chlamydia*, genetic diversity, Strzelecki Ranges, landscape change and management.

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Introduction

The present distribution and structure of Australian wildlife populations are the result of the events and environmental conditions of the recent and distant past. While environmental changes in the distant past occurred relatively slowly, a multitude of changes impacting Australian flora and fauna have been occurring rapidly since European colonisation (1788–present), resulting in the extinction of many species (Woinarski *et al.* 2015) and changes in the genetic structure of populations of surviving species (Tracy and Jamieson 2011; Frankham *et al.* 2012). Deforestation or 'land clearing' has been a major factor affecting Australian wildlife populations since European

settlement. Clearing of forests and woodlands occurred in order to allow the rapid development of agriculture from the mid-1800s up until to the mid-1900s (Bradshaw 2012). Extinctions and biodiversity losses may take a considerable length of time to occur following habitat disturbances such as land clearing (MacHunter *et al.* 2006; Vellend *et al.* 2006); species may therefore falsely appear to be unperturbed by landscape changes for a long time after degrading processes appear to have ended (Szabo *et al.* 2011). Even for species which remain relatively abundant, environmental changes can alter population structure and reduce the level of genetic diversity within populations,

so that apparently robust populations carry with them the legacy of past environmental pressures (Bijlsma *et al.* 2000). Genetic diversity is important for populations as it allows them to adapt to further changes and challenges which may emerge within their environment (Frankham *et al.* 2012), and therefore to persist, rather than becoming another entry on the list of extinct native Australian wildlife species.

Species that are habitat specialists are considered more prone to the negative impacts of environmental change than habitat generalists (Travis 2003). One habitat specialist, the koala (*Phascolarctos cinereus*) is an iconic Australian marsupial which exists on a near exclusive diet of eucalypt leaves. The distribution and genetic structure of koala populations is likely to have changed substantially since European settlement. In addition to the widespread clearing of habitat for agriculture, hunting in order to harvest pelts for the fur trade had a major impact on koala populations (Lewis 1934). In the future, rapid changes in climate are anticipated to result in contractions and spatial shifts in the distribution of most eucalypt species, which is of significant conservation concern for koalas (Adams-Hosking *et al.* 2011; McAlpine *et al.* 2015; González-Orozco *et al.* 2016). Continued habitat loss and forest fragmentation, due to urbanisation, industrial developments, forestry and fires have ongoing effects on contemporary koala populations, while the frequency and severity of droughts and fires, predicted to increase in Australia due to climate change (Hennessy *et al.* 2005; Bradstock 2010), will also continue to put pressure on this species. Climate change and increased atmospheric carbon dioxide may also alter the nutritional composition of eucalypt leaves, potentially reducing the suitability of some eucalypts as browse for koalas (Moore *et al.* 2004).

The movement of individual koalas from one region to another, particularly in south eastern Australia, has also affected the health (Santamaria and Schlagloth 2016) and genetic structure (Houlden *et al.* 1999; Lee *et al.* 2011) of koala populations. Early translocations of koalas (1860s–early 1900s), by individuals and acclimatisation societies (ASV 1872) were followed by more recent (1923–present) interventions by government agencies charged with population management objectives (Menkhorst 2004; Menkhorst 2008; DELWP 2015b). Thus, both environmental changes and translocations are likely to have impacted the present distribution and genetic structure of koala populations. The situation for the koala is now likely to be vastly different in terms of where, and how many, individuals can be found; and in the genetic composition of individuals and populations, compared to at the time of European settlement (McAlpine *et al.* 2015).

Koalas were once more broadly distributed across Australia. Fossils have been found on the Nullarbor Plain and on the southern coast of Western Australia (Black *et al.* 2014). The disappearance of the koala from these former parts of

its range (sometime after 43,000 years ago) is thought to have occurred due to expansion of shrub and grass land and contraction of forest and woodlands during the last glacial period, where rainfall decreased and seasonality and droughts increased (Price 2012; Black *et al.* 2014).

There is no fossil evidence that koalas ever occupied Tasmania (Price 2012; Black *et al.* 2014). This fact suggests that the southerly expansion of koalas into Victoria occurred relatively recently (Sherwin *et al.* 2000), after the inundation (around 10,000 years ago) of the Bassian Plain, which formed the land bridge between present day Victoria and Tasmania. It is also possible that more southerly cooler climates at the time rendered Tasmania unsuitable for koalas, though currently, parts of Tasmania are modelled as climatically suitable for koalas (Adams-Hosking *et al.* 2011).

South Gippsland

South Gippsland is a Victorian region located to the east of Melbourne (Figure 1). It includes the Bass Coast, South Gippsland, Latrobe City and Wellington shires and consists of three bioregions: the Strzelecki Ranges, the Gippsland Plain and the Wilsons Promontory bioregions (Figure 1B)¹.

Agriculture and forestry are the major land uses in the South Gippsland area and koalas use trees within both environments. The South Gippsland koala population is believed to be a remnant population; not derived from animals relocated as part of the Victorian koala translocation program (Menkhorst 2004; Lee *et al.* 2011).

The Strzelecki Ranges

One of the three bioregions in South Gippsland, the Strzelecki Ranges encompasses 3,500 square kilometres of land (Figure 1). Extant natural forest in the Strzelecki Ranges bioregion is of several major forest types: wet and damp forest at higher elevations, and lowland and shrubby foothill forest in the lower areas (DELWP 2015c). The western Strzelecki Ranges (from Western Port Bay in the west to approximately Mirboo in the east) is dominated by agricultural land, which was taken up and cleared by selectors during the second half of the 19th century.

The eastern end of the Strzelecki Ranges, extending from near Mirboo in the west through to the Carrajung area in the east covers an area of around 2,000 square kilometres (DELWP 2016). Ongoing land cover changes resulting from agriculture, forestry and wildfires in the eastern Strzelecki Ranges (Zhang *et al.* 2008) are likely to have had a substantial impact on koala populations

¹ In Australia, bioregions are both defined and used by state government agencies, and others, for biodiversity planning and land management purposes (DELWP 2015b). Bioregions are areas of land defined by similarities in geological and ecological characteristics.

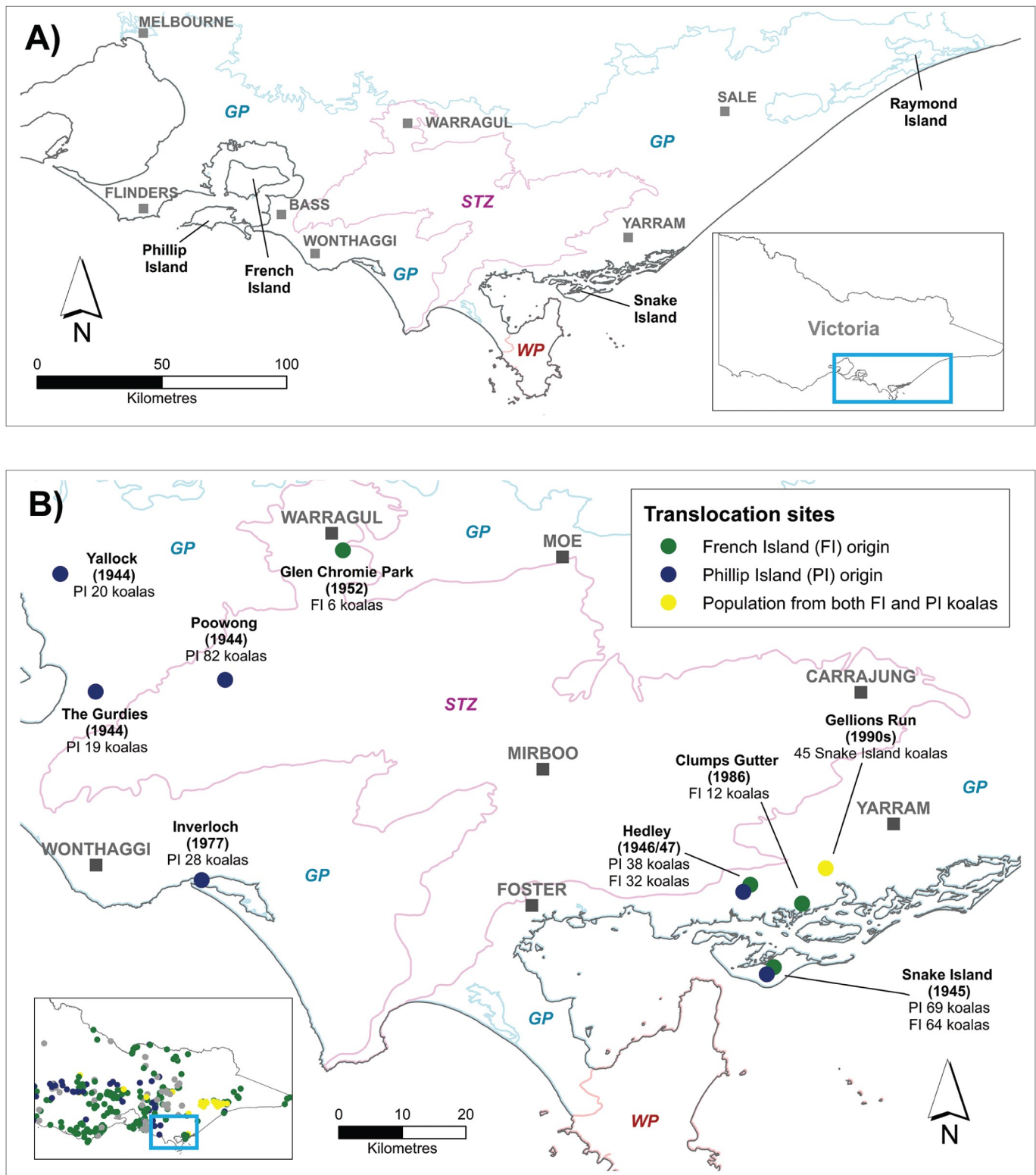


Figure 1. A) Map of southern Victoria showing the regions and islands discussed in the text. Bioregions are outlined in purple (STZ: Strzelecki Ranges bioregion), blue (GP: Gippsland Plain bioregion) and red (WP: Wilsons Promontory bioregion). B) Map of South Gippsland indicating sites and years that island koalas were translocated to the region. Grey points on the inset map of Victoria indicate translocations of koalas of unknown origin. The Victorian Bioregions dataset (DELWP, 2017) was obtained from www.data.vic.gov.au. Translocation data were obtained from Martin (1989) and Emmins (1996). Most individuals released from Snake Island onto the mainland were sterilised.

in this region. Land was first released in the Strzelecki Ranges by the government during the period from 1869 to 1874 (Legg 1986). At the time of settlement, koalas were reported to be “fairly numerous” and “plentiful” in the region (Elms 1920; Murray 1920; Williams 1920). As the land was settled, the forests of the eastern Strzelecki Ranges were cleared for agriculture² and by 1910 almost all of the land available for agricultural development in the ranges (approximately 1,800 square kilometres) was taken up (Legg 1986; Nelson *et al.* 2009).

Due to difficult terrain, erosion, the wet climate and infestations by weeds and introduced animals, many farms in the eastern Strzelecki Ranges failed and were ultimately abandoned (Legg 1986; Noble 1986). Farms were abandoned as early as 1882 but it wasn't until the late 1920s that farms began to be deserted on a large scale (Legg 1986). By 1930 there were more than 600 square kilometres of abandoned farmland and about 650 square kilometres of additional farmland in a serious state of neglect, representing close to 70% of the farms in the area (Legg 1986).

Following the mass abandonment of farms in the 1920s, further agricultural development in the ranges was not promoted by the government (Legg 1986) and the emphasis for the area became reforestation; the development of a timber resource (Noble 1986). The Forests Commission (a Victorian state government agency) began buying run down land in the early 1930s while APM Forests Pty Ltd (the forestry division of Australian Paper Mills, with a major milling operation in the nearby Latrobe Valley) began purchasing land in the 1950s. These land acquisitions were followed by the establishment of plantations across the region (Noble 1986).

Currently, around 650 square kilometres of forested land in the eastern Strzelecki Ranges is managed by HVP Plantations (HVP). The remaining area is mostly utilised for agriculture, but also contains numerous parks and reserves (EaCRC 2011b; DELWP 2016). HVP's estate consists of a matrix of plantations and native forest. The predominant species in the plantations are pine (*Pinus radiata*) and eucalypt species (*Eucalyptus regnans*, *E. nitens* and *E. globulus*); these are interspersed with around 250 square kilometres of native forest (EaCRC 2011b) managed for conservation purposes (HVP plantations 2015). Eucalypt species that dominate in native forest on the north of the range include Mountain Ash (*E. regnans*), Messmate (*E. obliqua*), Southern Blue Gum (*E. globulus*) and Mountain Grey Gum (*E. cypellocarpa*). On the south of the range Yellow Stringybark (*E. muelleriana*), Mountain Ash, Messmate and Mountain Grey Gum dominate in native forests.

Koalas inhabit the forests and plantations of the eastern Strzelecki Ranges. Past research on this population has been scant (EaCRC 2011a); so population structure, fragmentation and the rates of population growth or decline are not known. Preferred food species for koalas in the area are Mountain Grey Gum, Southern Blue Gum and Yellow Stringybark (Allen 2015). In areas containing these tree species, work carried out by HVP (R. Appleton, pers. comm. 2015, HVP) and Allen (2015) independently determined koala density estimates of 0.29 koalas per hectare and 0.25 koalas per hectare, respectively. Koala densities are much lower outside of these areas of good quality habitat (R. Appleton, pers. comm. 2015, HVP). The size of koala habitat trees is important to koala tree use; Allen (2015) demonstrated a preference for large mature trees of three highly favoured species (Mountain Grey Gum, Southern Blue Gum and Yellow Stringybark) in the Strzelecki Ranges, illustrating the importance of mature native forest to koala populations. A preference for larger trees has also been found for Manna Gum (*E. viminalis*) and Blackbutt (*E. pilularis*) in other areas (Santamaria *et al.* 2005; Matthews *et al.* 2007).

Fire has also played a role in environmental change in the eastern Strzelecki Ranges. Several major wildfires have affected the region since the mid-1800s. The first large bushfire documented to have impacted Victoria occurred in 1851, prior to the period in which the ranges were opened up for selection. A quarter of the state is believed to have burned at this time (Pyne 1991; DELWP 2015a). Anecdotal reports, from early settlers of the nearby Gippsland Plain indicate that the Strzelecki Ranges were widely burned by the ‘Black Thursday’ fires of 1851 and that the dense undergrowth, later encountered in the area, was a result of regrowth following these fires (Murray 1920). Subsequent major fires affecting the region included the Red Tuesday fires of 1898, which burned about 2,600 square kilometres throughout South Gippsland and the Black Friday fires of 1939, which affected an extensive area of the eastern Strzelecki Ranges (Pyne 1991; DELWP 2015a). Part of the eastern Strzelecki Ranges were also burned in the Black Saturday fires of 2009 which affected approximately 200 square kilometres of forest in the area surrounding Traralgon South, Koornalla and Callignee (DELWP 2015a).

In the 1920s and 1930s, koalas had become scarce in the Strzelecki Ranges and were believed to be very near to extinction (Williams 1920; Lewis 1954). However, during this time, surviving koala populations were reported at Wilsons Promontory (Kershaw 1928), Yarram (Lewis 1954) and around Carrajung and the foothills of the Strzelecki Ranges south of Morwell (Martin 1989). Ongoing changes in forest type and density in the Strzelecki Ranges since European settlement are likely to have had a continual impact on koala populations, resulting in a long history

2 One driving force of rapid land clearing in Australia were the Land Selection Acts of the 1800s which required that landholders made certain ‘improvements’ within their first two years of tenure (Nelson *et al.* 2009).

of population disruption and fragmentation. Since land clearing, forest regeneration and plantation establishment has occurred in various areas at different times for almost 150 years (1869–present); koala habitat in the Strzelecki Ranges is therefore likely to have locally shifted, appeared and disappeared since being opened up for selection.

In the mid to late 1800s, hunting of koalas by European settlers for the fur trade was widespread (Lewis 1934). In 1889 throughout Australia, 300,000 koala skins were reported to be exported for the year (Lydekker 1894) while in 1902 more than 600,000 koala skins were exported (*The Advertiser* 1902). Hunting for the commercial fur trade also occurred in the South Gippsland area with one Melbourne tannery reporting that their koala skins were mostly obtained from the South Gippsland region (*Weekly Times* 1896). In December 1898, koalas were provided protection in Victoria under the *Game Act 1890* (Victorian Government 1898). Purportedly however, hunting of koalas continued to some extent by exporting (both interstate and overseas), koala skins falsely labelled as ‘wombat’ in order to circumvent these protections (*The Argus* 1928; Jackson 2007).

The Gippsland Plain

The Gippsland Plain bioregion surrounds the Strzelecki Ranges bioregion (Figure 1A) and extends from the Mornington Peninsula in the west to Bairnsdale and Lakes Entrance in the east. It includes Phillip and French Islands in Western Port Bay. Altogether, the Gippsland Plain bioregion encompasses an area of about 12,000 square kilometres. On arrival to South Gippsland the first settlers are said to have seen a koala in almost every tree (Lewis 1952). After European settlement, deforestation initially occurred mainly in the fertile coastal areas (Bradshaw 2012) where pastoral runs were taken up from the 1840s; large scale conversion of forest to agricultural land is likely to have begun around 30 years earlier on the Gippsland Plain than in the Strzelecki Ranges. Early after settlement, Howitt (1890) described the Yellow Stringybark forest in the region as consisting of trees ranging from 30 to 60 metres in height and covering an area of around 800 square kilometres, extending from the foothills of the Strzelecki Ranges towards the coast³. Within the Strzelecki Ranges, koalas are currently found at relatively high densities in remnant vegetation dominated by Yellow Stringybark (R. Appleton, pers. comm. 2015, HVP; Allen 2015). Due to conversion to farmland, the majority of the Yellow Stringybark forest on the Gippsland Plain no

longer exists. Substantial impacts on koala populations would have resulted from the widespread loss of this important food tree in the region. Settlers on the Gippsland Plain did not face the same difficulties as those in the eastern Strzelecki Ranges; agriculture has been successful there and the area is a major dairy centre in Victoria (Agriculture Victoria 2014).

Wilsons Promontory

The third of the South Gippsland Bioregions is Wilsons Promontory, a headland, connected to the Victorian mainland by a low narrow isthmus (~ 8 kilometres wide; Yanakie Isthmus). It extends into Bass Strait forming the southernmost part of the Australian mainland. Wilsons Promontory covers an area of over 400 square kilometres and is located south of the Strzelecki Ranges and Gippsland Plain bioregions (Figure 1). Koalas were once very abundant on Wilsons Promontory and were reportedly common around Oberon Bay, Sealers Cove, Five Mile Beach, Barry Creek, the Darby River area and on the Yanakie Isthmus (Meagher and Kohout 2001; Garnet 2009). Koalas were apparently so common on the promontory that they could be seen “in nearly every manna gum” with “several” often being seen in a single tree (Barrett 1939). Currently, the density of the Wilsons Promontory koala population appears extremely low, and koala sightings are rare (J. Whelan, pers. comm. 2013, Parks Victoria). Reasons for recent decline of the Wilsons Promontory koala population are not clear though the area has undergone a variety of land use and land cover changes since European settlement.

Cattle and sheep were once grazed on the promontory, with pastoral leases encompassing most of Wilsons Promontory from the 1850s (Garnet 2009). During the 1800s, whaling settlements were built at Sealers and Refuge Coves, and Tin Mine Cove was settled by workers mining tin at Mount Hunter (Meagher and Kohout 2001). The forests around Sealers Cove were heavily logged in the 1840s and 1850s as well as in the early 1900s (Garnet 2009). During World War II, a military camp was established at Tidal River with training exercises frequently occurring throughout the park (Meagher and Kohout 2001). Wilsons Promontory was declared a National Park in 1908, though grazing licences were still granted through to the 1970s (Meagher and Kohout 2001). Many plant and animal species were introduced to Wilsons Promontory in the late 1800s and early 1900s (Garnet 2009), including hog deer, emus and kangaroos, which continue to thrive there (Meagher and Kohout 2001; Whelan 2008). Numerous fires have affected the promontory since European settlement, with major wildfires occurring in 1863, 1907/08, 1921, 1939, 1943 and 1951 (Meagher and Kohout 2001; Garnet 2009). Due to its isolation, being largely bounded by sea, fires on Wilsons Promontory are likely to have had a severe impact on wild animal populations, since post-fire recolonization opportunities are limited.

3 In the foothills of the Strzelecki Ranges (on HVP estate), the area currently covered by forest dominated with Yellow Stringybark is around 13 square kilometres. The Won Wron and Mullungdung State Forests to the east of the Strzelecki Ranges (on the Gippsland Plain) encompass about 175 square kilometres of Plains Grassy Woodland (EVC 151) that is often dominated by Yellow Stringybark and/or Messmate and may also be important koala habitat.

In the late 1800s and early 1900s, the koala population on Wilsons Promontory was at a high density (Barrett 1939); hunters were purportedly able to obtain 2000 koala pelts from the promontory in a single year (Hardy 1906). A “diminishing quantity” of koalas was noted by Hardy (1906) and attributed to predation by dingoes and wild dogs for which over 100 strychnine baits were laid by the Field Naturalists Club of Victoria. During 1915–1918 it was reported that koalas were overpopulating Frasers Creek, near Oberon Bay (Kershaw 1915; Hardy 1918) and the flats north of Darby River (Barrett 1939). The flats near Darby were, however, previously forested with “fair sized eucalypts” in which koalas were common, but by 1913, the trees were reported to have been killed by ringbarking⁴ (Kershaw 1913). Localised overpopulation of koalas at Wilsons Promontory resulted in the defoliation and death of many of their remaining food trees (Barrett 1939; Menkhorst 2008). In order to prevent the starvation of many animals, koalas were captured and moved to other regions of the promontory, given to wildlife societies across the country and culled (Hardy 1918; *The Argus* 1939). Koalas have persisted on Wilsons Promontory but have not increased to the high levels observed in the early 1900s. The population is currently at very low density illustrating that overpopulation does not necessarily guarantee long term population security.

Pre-European South Gippsland

Humans have inhabited the Australian continent since the arrival of Aboriginal Australians at least 60,000 years ago (Roberts *et al.* 1994; Malaspinas *et al.* 2016). At the time of European settlement, South Gippsland was home to the Brataualung clan of the Gunaikurnai people (Figure 2) (Fison and Howitt 1880; Gunaikurnai Traditional Owner Land Management Board 2016). In the east of the South Gippsland region, the Brataualung people are believed to have lived mainly on the plains near the coast where food was plentiful; the forests of the Strzelecki Ranges were less commonly used as they were too wet, and supported less abundant food resources (Morgan 1997; Gott 2005). Neighbours to west of the Brataualung, were the Bunurong people who lived around Western Port Bay and eastwards into South Gippsland (Wesson 2000; Ellender 2002).

Koalas in South Gippsland were hunted on occasion by Aboriginal people, though to what extent is not well documented (Fison and Howitt 1880; Howitt 1904). Aboriginal people are known to have used fire to promote the availability of food resources, both plant and animal, although there is little information on fire use and regimes locally (Gott 2005; Gott *et al.* 2015). Cahir *et al.* (2016) noted that most historical accounts of Aboriginal burning practices in south-east Australia (including Gippsland)

indicate that the application of fire, “was managed, was frequent and was generally over small areas of grassland plains”. Conversely, burning of wet sclerophyll forest (such as parts of the Strzelecki Ranges) is not considered likely, especially given the scarcity of food plants in such forests (Gott 2005).

By 1860, the Aboriginal population had decreased dramatically (Gardner 1993). Historic evidence indicates a major cause of decline to be the widespread murder of Aboriginals by the settlers and numerous massacres organised and led by early settler and explorer, Angus McMillan (Gardner 1993). A widespread lack of burning by Aboriginal people in the post-colonial period (Gardner 1993) is likely to have resulted in changes to habitats and their ecosystems that may subsequently have had an impact on resident wildlife populations. For example, in 1840, explorers noted the presence of thick scrub on the Gippsland Plain (Horton and Morris 1983; Morgan 1997) which has been suggested to be growing vigorously on lands previously burned by Aboriginal people, but left unmanaged after their displacement some years before (Ellender 2002). Additionally, localised tree dieback was attributed, by Howitt (1890), to the cessation of firing by Aboriginal people resulting in an increase in insect populations and insect attack.

Early translocations

The Acclimatisation Society of Victoria (ASV) was formed in 1861 with the major aim of introducing foreign “innocuous” plant and animal life to Victoria (ASV 1861). Additional aims included exchanges of live animal specimens with other countries and “the spread of indigenous animals from parts of the colonies where they are already known, to other localities where they are not known” (ASV 1861). The ASV advertised requests for donations of native animals (*The Argus* 1861), including “native bears” (koalas), which were caught and “donated” to the ASV. Little was known about koala husbandry and koalas were difficult to keep in captivity (Le Souef 1878); numerous early attempts to send live koalas to societies overseas failed, resulting in the deaths of these animals (Jackson 2007) and by the 1870s requests for donations of native animals specifically excluded koalas (ASV 1871). A search of Victorian newspapers between 1861 and 1894 found reports of 134 koalas donated to the ASV, with many occurring after the 1870s (Appendix, Table A1); it is therefore clear that receipt of these animals continued despite their exclusion from advertisements. Ferdinand von Mueller, government botanist and member of the ASV reported that koalas were sent to Hobart Town, Tasmania in 1862 (three koalas; *The Leader* 1862), 1864 (eight koalas; *The Argus* 1864) and 1872 (nine koalas; ASV 1872) and to South Australia in 1864 (five koalas; *The Leader* 1864). Apart from the few releases listed above, evidence indicating the fate of the many koalas donated to the ASV were not located. Though undocumented,

⁴ Ringbarking describes the cutting away of bark around a tree trunk in order to kill the tree. Ringbarking was an often-employed method for clearing trees for agricultural purposes.

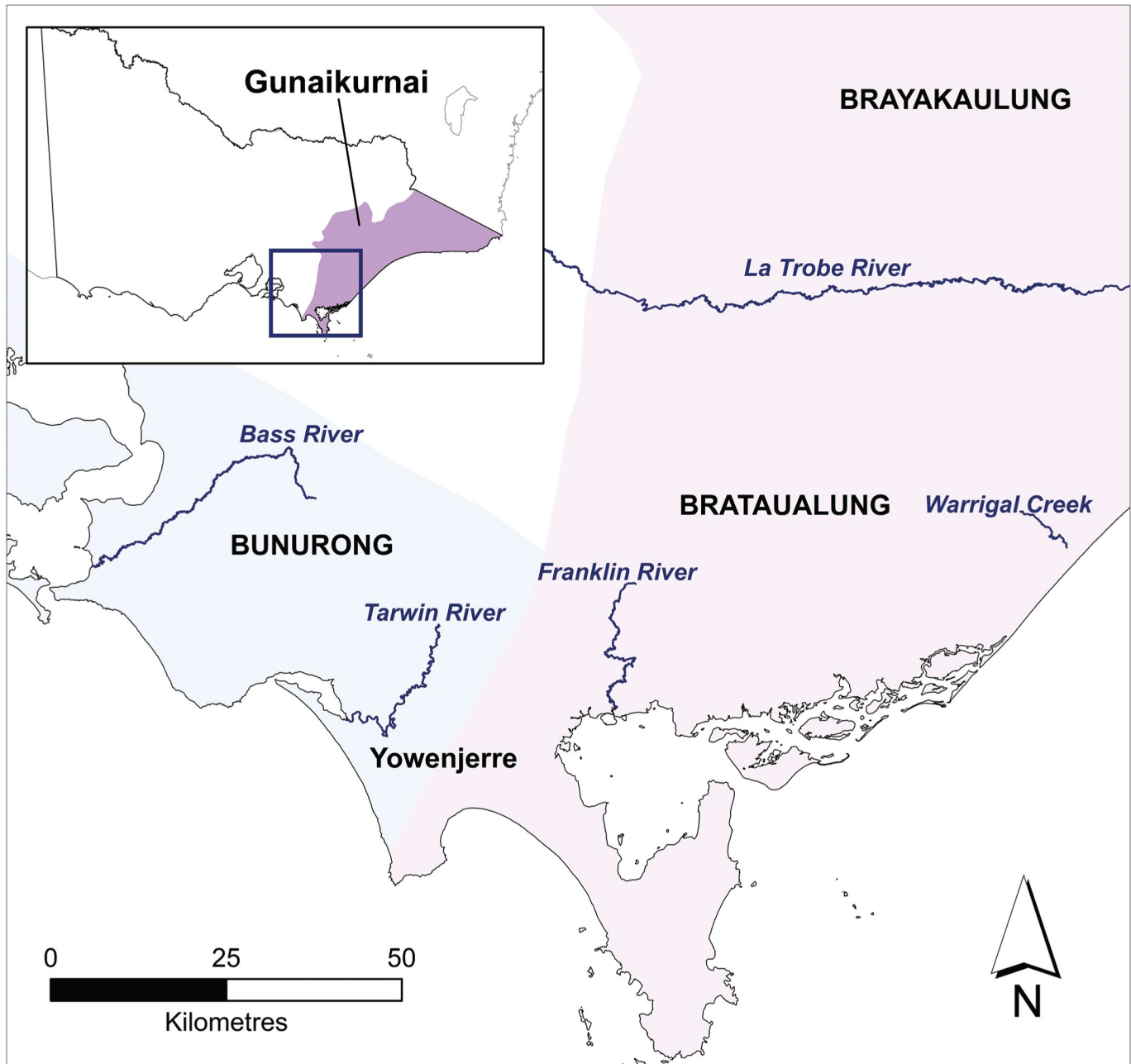


Figure 2. Aboriginal clans in the South Gippsland region. Adapted from Fison and Howitt (1880), Ellender (2002) and Gaughwin and Sullivan (1984).

numerous koalas are likely to have been transferred from one area to another via the ASV and their donors, as well as by other individuals or groups. In 1911, for example, while in the Wonthaggi district (on the Gippsland Plain), Nicholls (1911) reported having heard that thirty koalas had been sold to travellers from a nearby train station platform at Christmastime for half a crown each.

French and Phillip Islands

Phillip and French Islands in Western Port Bay were not inhabited by koalas at the time of settlement. The current French Island koala population is believed to have been founded by a single release of a small number of koalas

taken to the island by fishermen from Corinella in the late 1800s (Figure 3; Lewis 1954). In *The Argus* (1924), J. G. Palmer from Corinella stated that in around 1898–1900 his brother, F. Palmer took two “old” koalas and one “young” koala from the mainland and released them on French Island. Genetic evidence suggests that a minimum of three individuals (Houlden *et al.* 1996) founded the French Island population: two females and one male (Taylor *et al.* 1997).

The Phillip Island koala population was established by a larger number of individuals than the three released on French Island with introductions occurring on more than one occasion (Figure 3; Lewis 1954). John McHaffie

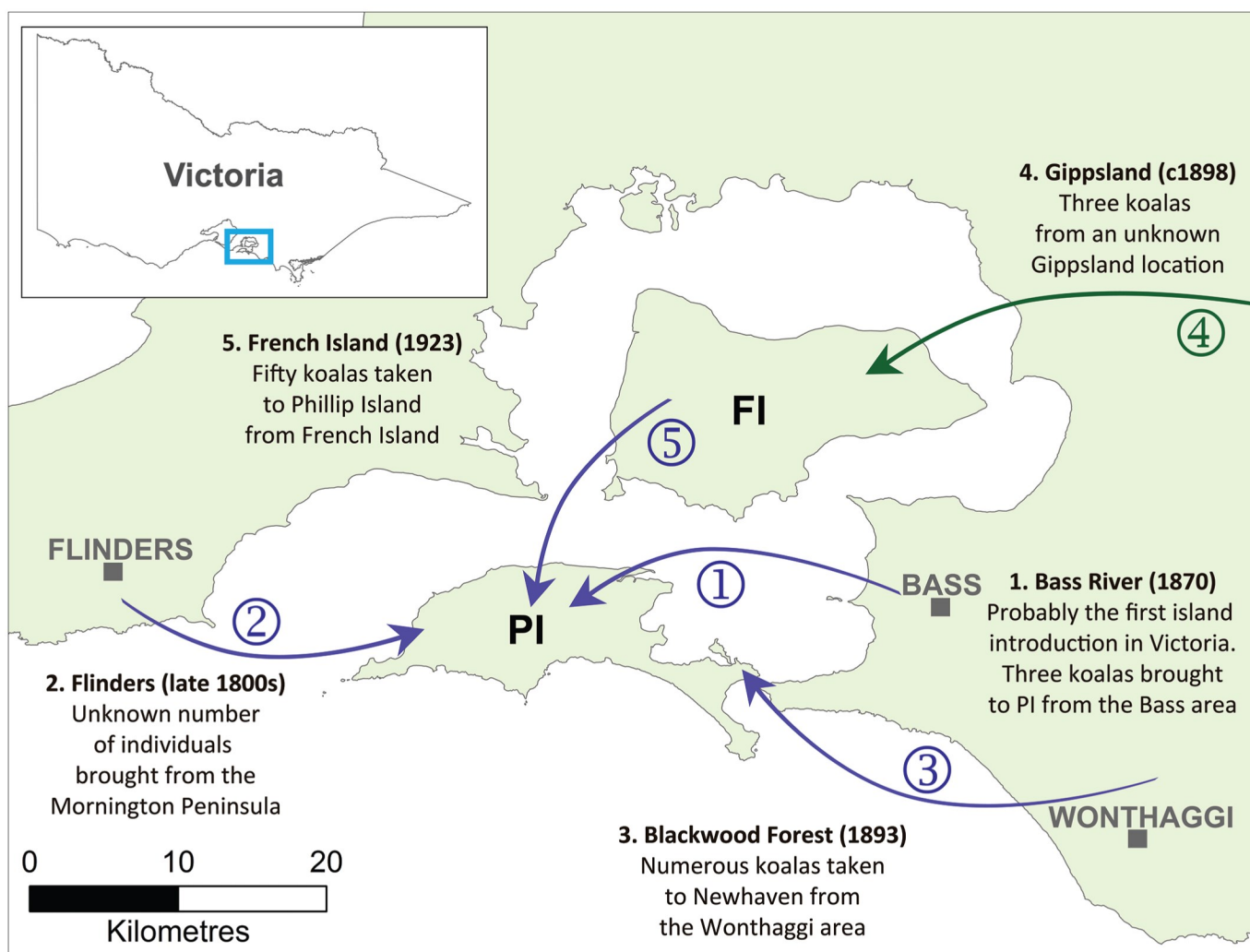


Figure 3. Map illustrating the 1800s history of koalas moved to French Island (FI) and Phillip Island (PI) in Western Port Bay, as described in the text, whose descendants were to become the source of most contemporary Victorian koala populations.

was the sole occupant of Phillip Island from 1848–1868 a dedicated member of the ASV, breeding and releasing many animals on Phillip Island for the society (Wright 1980), though no evidence was located to suggest that koalas were ever released on Phillip Island by the ASV. Koalas were reportedly taken to Phillip Island by early pioneers (Gliddon 1958; Edgecombe 1989) and were brought from Bass River, by J. F. Smith, in 1870 (Lewis 1954; Gliddon 1958); from Flinders (on the Mornington Peninsula), by W. Kennon (Lewis 1954); from Blackwood Forest (near Wonthaggi), by C. and R. Grayden in 1893 (The Age 1938; Lewis 1954); and from French Island, by W. E. Thompson in 1923 (Lewis 1954).

Gliddon (1958) describes Mr John F. Smith's recollection of hunting for wallabies at Bass River in 1880 with his brother, George, and Bill and Jack Walton. During this hunting trip, three koala joeys were captured and brought back to Phillip Island. Richard Grayden reported, in a letter to the editor in *The Age* (1938), that as a teenager, he had captured dozens of koala joeys from Blackwood Forest (near Wonthaggi). These, he took home to Newhaven,

Phillip Island, where “every child living there had one for a pet” and “after a few days they were allowed to climb a tree, and soon gained their freedom”. Richard Grayden stated that it was after this time that the koala population increased and spread across the entire island and also mentions that the introduction of 50 French Island koalas to Phillip Island by Mr. Thompson in 1923 was prompted by a particularly wet winter which resulted in the death of many koalas on Phillip Island (The Age 1938).

Unsustainable koala densities can occur where habitat is isolated and/or animals are unable or unwilling to disperse (Whisson *et al.* 2016), often resulting in over browsing and death of food trees, which may subsequently lead to the starvation of individual animals. Increased population densities therefore often necessitate the relocation of animals to other regions to prevent the death of both trees and koalas (Menkhorst 2004; DELWP 2015b). By the 1920s and 1940s, the koalas released on French and Phillip Islands, became established to the point of unsustainability; the population density having increased above the carrying capacity of the islands.

Re-establishing koala populations across Victoria

In 1924, 25 years after the initial release of as few as three koalas on French Island, it was reported that around 20 koalas could be counted along a 400 metre stretch of forested road (*The Argus* 1924). At roughly the same time, the first documented translocation, as mentioned in the preceding section, was undertaken (by W. E. Thompson), where, assisted by schoolchildren, 50 French Island koalas were captured and released on Phillip Island (*Frankston and Somerville Standard* 1924; Martin 1989). The first translocations from Phillip Island were carried out in 1941 (Martin 1989). Such translocations were intended to solve the growing problem of koala overpopulation on the islands as well as to assist koala conservation by re-establishing mainland koala populations (Menkhorst 2008).

Since the state government translocation program began in the 1920s, koalas have been released at hundreds of different sites across Victoria⁵ (Menkhorst 2008). Over 8,500 koalas were translocated from French Island between 1923 and 2006 (Menkhorst 2008), while approximately 3,500 Phillip Island koalas were translocated to various sites across Victoria between 1941 and 1978 (Martin 1989). Since 1978, however, the Phillip Island koala population has declined considerably to an estimated 13 individuals in 2006 (EaCRC 2011a), again illustrating how population size can change dramatically over a relatively short time span. The re-establishment of koala populations across Victoria, through the translocation program, has been claimed to have been so successful that koalas are currently occupying almost all suitable habitat in the state (Menkhorst 2008). A history of koala management and the translocation program in Victoria has been thoroughly reviewed by Menkhorst (2008) and reveals that most koala populations in Victoria are descended from the small numbers of founding individuals initially introduced to French and Phillip Islands.

It is unlikely that the full breadth of koala translocations is known, given undocumented translocations by the ASV in the late 1800s, and since then by the government, wildlife carers (Guy and Banks 2012) and other individuals. Documented translocation data may also be incomplete or have inaccuracies (e.g. Hogan *et al.* 2013). For example, official translocation records indicate that six and twelve French Island koalas were translocated to Kangaroo Island, South Australia in 1923 and 1925, respectively (Martin 1989). A report in *The Argus* (1923), however, reports that the first six

koalas translocated to Flinders Chase, Kangaroo Island in 1923 were from Wilsons Promontory National Park (rather than French Island) which was at the time highly overpopulated (Kershaw 1915; Hardy 1918; Barrett 1939). Genetic data also provides evidence that the documented source population for the first Kangaroo Island translocation may be inaccurate; alleles and haplotypes not present in the French Island population have been detected in the Kangaroo Island population (Houlden *et al.* 1996; Cristescu *et al.* 2009; Cristescu *et al.* 2010; Neaves *et al.* 2016). The Kangaroo Island koala population may, therefore, have been established by a broader subset of koalas than from French Island alone. Findings such as these reinforce the need to confirm historic records using molecular methods, where required, in order to ensure that management decisions are based on the most accurate information possible.

Genetic diversity in Victorian koala populations

Although government translocations (1923–present) of koalas within Victoria have been successful in terms of re-establishing koala populations across the state, the translocations that followed the decimation of Victorian mainland koala populations are likely to have had an overall negative impact on the genetic diversity of Victorian koalas. By the 1920s the koala was nearing extinction (Lewis 1954) and the gene pool already narrowed. A state government investigation at the time estimated that the koala population on the Victorian mainland had been reduced to around 500 to 1000 individuals, predominantly located in and near to the Strzelecki Ranges (Lewis 1934; Lewis 1954; Martin 1989; Menkhorst 2008). The Victorian situation was mirrored in other states at this time. In the 1920s there were an estimated 10,000 koalas remaining in Queensland, 200 in New South Wales and none in South Australia⁷, though whether koalas were widely distributed in South Australia at the time of European settlement is not clear⁸ (Phillips 1990). This represented a considerable reduction in population size and hence a loss of genetic diversity (known as a genetic bottleneck) for koala populations at a national scale.

When populations decrease in size, there is an initial loss of genetic diversity and a possibility of continued loss across future generations due to chance, in a process called genetic drift (Frankham *et al.* 2012). Small population sizes also increase the chance of inbreeding which can further exacerbate losses of genetic diversity (Frankham *et al.*

5 Koala translocations continue to present day (2017) in order to manage the size of some Victorian koala populations

6 These figures represent official documented translocations and therefore the minimum number of individuals translocated. It is possible that further official translocations occurred for which documents were not kept or have been lost. More than 12,000 additional individuals have also been translocated from other populations established by the translocation program (Menkhorst 2008).

7 Although the koala is considered a single species (Houlden *et al.* 1999), documented translocations across state borders have not been common and may have been bureaucratically difficult.

8 It is stated in Lewis (1952) that “when white people first came to Australia the koala was exceedingly abundant in the three eastern mainland States, even extending into South Australia at the south-east corner, along the Glenelg Valley”.

al. 2012). In addition, when a small subset of individuals separate and establish a new population (either naturally or via translocation), genetic structure can be altered further. These changes are termed ‘founder effects’ by population geneticists (Frankham *et al.* 2012).

The founder effect is relevant to many modern Victorian koala populations. The translocation of small numbers of Gippsland koalas to French and Phillip Islands is an example. Since the numbers of founding individuals for both island populations were small, further losses of genetic diversity due to inbreeding and chance (genetic drift) may have occurred within the island populations. Because these island populations were subsequently used as source populations for additional translocations, to re-establish koala populations in other areas of Victoria, the situation has been exacerbated. Mainland koala populations founded by island individuals have undergone at least two genetic bottlenecks within 100 years, with some populations having undergone multiple founder events, potentially resulting in even greater losses of genetic diversity.

The importance of genetic diversity

Genetic variation is important as it provides populations with the capacity to adapt and survive environmental changes, while decreased variation negatively affects survival, growth and reproduction rates (Sherwin *et al.* 2000; Frankham *et al.* 2012). Species that have been subjected to bottlenecks in the past are at a greater risk of extinction, even when numbers of individuals within populations recover (Bijlsma *et al.* 2000). Levels of genetic variability can therefore be more important than abundance for a population’s future viability. For example, the Tasmanian devil (*Sarcophilus harrisii*) was abundant in Tasmania prior to 1996, despite its history of bottlenecks and low levels of genetic diversity (Siddle *et al.* 2007). Since that time, Tasmanian devil populations have decreased by between 50% and 90% due to the emergence of the highly contagious, devil facial tumour disease (Siddle *et al.* 2007). Low genetic diversity is thought to have resulted in a reduced ability of the Tasmanian devils’ immune systems to recognise and destroy tumour cells (Siddle *et al.* 2007; Woods *et al.* 2007). This is not to say, however, that species with low genetic diversity do not have a future in the long term, as there are examples of species surviving for relatively long periods despite low genetic diversity (Reed 2010). The risk of extinction due to low diversity also depends on life history, stochastic factors and the interplay between them (Reed 2010). Certain Victorian koala populations with low levels of diversity are currently thriving, however, there is a chance that these populations are yet to be subjected to pressures to which they may not be able to cope with because of their limited gene pool. Such a situation is likely to result in population declines. There is therefore

potential for low diversity koala populations to undergo rapid decline due to future pressures such as epidemics, changes in climate and/or further loss or fragmentation of suitable habitat.

Chlamydia associated risks

A disease which afflicts some koala populations is chlamydiosis. It results from a bacterial infection with *Chlamydia* and can affect the urinary and reproductive tracts, sometimes rendering female koalas sterile. *Chlamydia* can spread quickly through populations. Translocation of *Chlamydia* free animals to habitat containing resident *Chlamydia* positive individuals was found to result in the infection of 13/14 animals tested after 19 months (Santamaria and Schlagloth 2016). This study did not observe any overt signs of infection, however, breeding success went from 6/16 in the first breeding season to 1/16 in the second breeding season. *Chlamydia* is not currently widespread in koala populations derived solely from French Island (Emmins 1996; Legione *et al.* 2016a; Legione *et al.* 2016b) and, on exposure, these koalas are sometimes found to be more susceptible to severe infections (Martin and Handasyde 1990). Therefore, koala populations derived from the historical French Island population could be at greater risk of future declines and extinction if urogenital infections with *Chlamydia* were to become prevalent in these populations. This situation is suggested to have occurred for the koala population in the Grampians National Park, established by the translocation of 611 French Island (*Chlamydia* negative) individuals in 1957 (Martin 1989; Martin and Handasyde 1999). The Grampians population quickly increased, but crashed in the 1970s (Menkhorst 2008) and afterwards remained at much lower densities (Martin and Handasyde 1999). A survey of the Grampians population in 1986/7 identified high levels of infection and a fertility rate of zero (Martin and Handasyde 1990). The decline in the Grampians koala population has been suggested to be due to the release of 60 *Chlamydia* positive koalas⁹ translocated to the Grampians National Park in 1963 and the subsequent spread of *Chlamydia* throughout the naïve population (Martin and Handasyde 1999).

Population genetic studies in the South Gippsland region

The history and geography of landscapes in South Gippsland landscape are thought to have permitted the survival of a relatively substantial koala population at the time when most other Victorian koala populations had severely declined or were extirpated (Lewis 1954; Houlden *et al.* 1999; Menkhorst 2008; Lee *et al.* 2011). The South

⁹ The 60 koalas were from Wartook Island in the Grampians. This population had been founded by stock from Phillip Island and the Creswick koala reserve (the latter originally established using Phillip Island koalas; Martin 1989).

Gippsland koala population has also remained somewhat separated from other Victorian koala populations. Current government policy prohibits the release of island-derived animals in South Gippsland (Menkhorst 2004), though several early translocations from island populations to South Gippsland have occurred (Figure 1B). The extent to which island individuals may have integrated with local populations is unknown, though genetic studies show that remnant diversity exists in South Gippsland indicating that complete genetic swamping of local diversity by translocated island animals did not occur (Lee *et al.* 2011; F. Wedrowicz, unpublished).

Genetic studies have shown that the South Gippsland koala population, which includes koalas in both the Strzelecki Ranges and Gippsland Plain bioregions, is genetically different from, and more diverse than, island populations and their descendants (Emmins 1996; Houlden *et al.* 1999; Lee *et al.* 2011; F. Wedrowicz, unpublished). The greater genetic diversity of the South Gippsland koala population could provide it with a greater chance of survival, compared to island derived populations, when challenged with future environmental changes. The potential importance of the South Gippsland koala population is recognised within Victoria (Martin 1989; Menkhorst 2004), though comprehensive surveys of genetic diversity in South Gippsland and throughout Victoria, to identify other potential remnant populations, are lacking. Koala population trends in South Gippsland are also unknown and there is a need for such data to inform appropriate conservation strategies.

Conclusions

After European settlement, widespread landscape modification occurred rapidly, resulting in irreversible changes to landscapes and their flora and fauna. Species extinctions, and, in surviving species, the loss of biodiversity at genetic levels, are effects that cannot be undone. This article has discussed a range of past anthropogenic and environmental impacts, which have occurred over the last two centuries and which have affected the South Gippsland koala population. Further genetic studies in the region can provide us

with key understandings that will help us to gain a more thorough appreciation of past events and their effects. The management of overabundant koala populations, in order to protect habitat and prevent widespread starvation of individual koalas, has been a key focus for the Victoria government. Of some concern for these populations, is that large population sizes may not be sufficient to evade the problems associated with low genetic diversity in the future. Conservation of the South Gippsland koala population and its genetic diversity is important because its higher genetic diversity may increase this population's future viability relative to other Victorian populations (Menkhorst 2004). The morphological (e.g. Briscoe *et al.* 2015) and genetic diversity (F. Wedrowicz, unpublished) present in South Gippsland koalas' represents a unique subset of the total diversity present across the species' range. The success of any one population in the face of future environmental changes is not known; some populations may persist while others may become extirpated. Conserving populations and their genetic diversity across the entire range of a species is important to minimise the risk of extinction. To date, research and data collection for Victorian koala populations has focussed on the few translocated populations that have become overabundant and require management. Information for a larger number of populations, particularly those with high levels of remnant genetic diversity, is needed for a successful approach to koala management and conservation. Studies to identify additional populations with high genetic diversity, to understand genetic relationships within and between populations and to monitor population size and impacts of disease are key actions required for future koala conservation.

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APPENDIX I

List of koalas reported to have been donated to the Acclimatisation Society of Victoria between 1861 and 1894. This summary is likely to be far from complete but gives an indication of the number of koalas that were purportedly removed from the wild and donated to the Acclimatisation Society in the late 1800s.

Date	Paper	Page	No. koalas	Donated by	Of
16/11/1861	<i>The Argus</i>	4	1	Mr. Trenchard	St Kilda
28/3/1862	<i>The Age</i>	5	1	Mr. Saint	Russell St
19/6/1862	<i>The Argus</i>	5	1	Mr. Richard Vinicombe Dennis	Birregurru
25/9/1862	<i>The Age</i>	4	1	Mr. W. Watson	Wooling, near Gisborne
30/10/1862	<i>The Argus</i>	5	1	Mr. Fleming	Plenty
30/10/1862	<i>The Argus</i>	5	2	Mr. John Mason	Belfast
20/11/1862	<i>The Age</i>	5	2	Mr. Feehan	City Arms Hotel
18/12/1862	<i>The Age</i>	4	1	Mr. Durrell	Gisborne
18/12/1862	<i>The Geelong Advertiser</i>	5	1	Mr. Landells	
8/1/1863	<i>The Argus</i>	4	1	Messrs J & R Waugh	Queensberry St
8/1/1863	<i>The Argus</i>	4	1	Mr. Hutton	North Melbourne
15/1/1863	<i>The Bendigo Advertiser</i>	2	1	Mr. Collie	Carlton
16/4/1863	<i>The Argus</i>	5	1	Mr. Mulcahey	
26/11/1863	<i>The Age</i>	5	1	Mr. R. T. Firebrace	Heyfield, Gippsland
31/12/1863	<i>The Age</i>	5	1	Mr. Bagshawe	Eltham
31/12/1863	<i>The Argus</i>	6	1	Mr. P. C. Borkey	Richmond
21/1/1864	<i>The Age</i>	5	1	Mr. J. Harvey	Woodend
18/2/1864	<i>The Argus</i>	2	1	Mr. Henry Howard	Schnapper Point
4/3/1864	<i>The Farmer's Journal and Gardener's Chronicle</i>	12	1	Mr. Tom Chew	Princess St, Fitzroy
10/3/1864	<i>The Argus</i>	5	1	Mr. Purvis	Richmond
14/4/1864	<i>The Age</i>	4	2	Miss Ellis Ryan	Brighton
12/11/1866	<i>The Geelong Advertiser</i>	2	1 white	Mr. J. Connor	M.L.A Colac
9/1/1867	<i>The Age</i>	5	1	Mr. D. C. Macarthur	Heidelberg
23/3/1867	<i>The Australasian</i>	3	1	Mrs. Hubbard	via Geelong branch
23/3/1867	<i>The Australasian</i>	3	1	Mr. Bedgegood	via Geelong branch
31/5/1869	<i>The Geelong Advertiser</i>	2	1	Mrs. Blackwood	
16/1/1871	<i>The Geelong Advertiser</i>	2	1	Mr. W. Higgins	
20/11/1872	<i>The Argus</i>	5	1	Mr. Baxter jun.	Frankston
4/12/1872	<i>The Age</i>	2	1	Mr. Robertson	Hotham
18/12/1872	<i>The Argus</i>	15	1	Mr. Edgar Slade	Alberton
1/2/1873	<i>The Australasian</i>	21	1	Mr. Harding	Maldon
24/9/1873	<i>The Age</i>	2	1	Mr. W. Robertson	Wooling

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Date	Paper	Page	No. koalas	Donated by	Of
10/3/1873	<i>The Ballarat Star</i>	2	1	Mr. Pickering	Smeaton
8/7/1874	<i>The Age</i>	2	1	Mr. Lewellyn	Prahran
25/11/1874	<i>The Argus</i>	6	1	Mr. William Lyall	Hazelwood
1/9/1875	<i>The Argus</i>	5	2 young	Mr. Ledger	Longwood
29/9/1875	<i>The Argus</i>	5	1		Chitern & Murray Acclimatisation Society
27/10/1875	<i>The Argus</i>	5	1	Mr. George Black	Norfolk Hotel, Collingwood
8/12/1875	<i>The Argus</i>	5	1	Mr. Batts	Yarra Flats
8/12/1875	<i>The Argus</i>	5	2	Mr. Oliver	Coliban Park
16/2/1876	<i>The Argus</i>	5	1	Mr. Godfrey	Mt Ridley
25/4/1877	<i>The Argus</i>	5	1	Mr. Murdoch	Wangaratta
13/4/1878	<i>The Australasian</i>	19	1	Mr. C. Tuck	Brighton
25/10/1878	<i>The Age</i>	3	1 white	Messrs. Griffiths and Gaunt	Bourke St
25/10/1878	<i>The Age</i>	3	1	Mrs. E. M. James	Collins St East
17/1/1879	<i>The Argus</i>	5	1	Mr. J. Sweetman	Carlton
26/3/1879	<i>The Argus</i>	5	1	Mr. Ballanger	Carlton Brewery
19/12/1879	<i>The Age</i>	3	1	Mr. E. C. Clark	Penal Dept., Melbourne
28/1/1880	<i>The Age</i>	3	1	Mr. Mindah	Hotham
28/1/1880	<i>The Age</i>	3	1	Mr. Simpson	Carlton
21/8/1880	<i>The Australasian</i>	19	1	Mr. H. O. Rosson	Bunyip
8/9/1880	<i>The Argus</i>	5	2	Mr. French	
8/9/1880	<i>The Argus</i>	5	1	Miss Annie Stewart	Western Port
8/9/1880	<i>The Argus</i>	5	2	Mr. Saunders	Mickleham
8/10/1880	<i>The Argus</i>	5	2	Mr. French	Christmas Hills
22/10/1880	<i>The Argus</i>	5	1	Mr. A. Miller	Carlton
20/11/1880	<i>The Australasian</i>	19	1	Mr. Coulthard	Carlton
20/11/1880	<i>The Australasian</i>	19	1	Mr. R. W. Blythman	Benalla
20/11/1880	<i>The Australasian</i>	19	1	Mr. R. Ralston	Wandong
29/1/1881	<i>The Australasian</i>	21	1	Mr. Max Straubel	Richmond
26/3/1881	<i>The Age</i>	5	1	Mr. Greenwood	
6/5/1881	<i>The Argus</i>	5	1	Mr. John Howlett	Hotham
6/5/1881	<i>The Argus</i>	5	1	Mr. C. E. May	Melbourne
20/5/1881	<i>The Age</i>	2	1	Mrs. Evans	Hotham
1/6/1881	<i>The Age</i>	3	1	Mr. Turner	Melbourne
30/6/1881	<i>The Argus</i>	5	1	Mr. McKellar	Strathkellar
26/1/1882	<i>The Argus</i>	7	1	Mr. Mowling	Windsor

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Date	Paper	Page	No. koalas	Donated by	Of
9/3/1882	<i>The Age</i>	3	1	Mr. G. A. Tyler	South Yarra
19/4/1882	<i>The Argus</i>	9	1	Mr. W. F. McFee	Hawthorn
4/10/1882	<i>The Argus</i>	5	1	Mr. John Johnson	Ascotvale, Flemington
18/10/1882	<i>The Argus</i>	5	1	Mr. A. Browning	Batman's Hill
11/1/1883	<i>The Age</i>	3	1	Mr. Thompson	Dandenong
11/1/1883	<i>The Age</i>	3	1	Mr. Bondle	Royal Park
22/8/1883	<i>The Argus</i>	11	2	Mr. John McMahon	Fitzroy
12/12/1883	<i>The Argus</i>	11	1	Mr. E. Baldwin	Kyneton
12/12/1883	<i>The Argus</i>	11	1 white	Mr. A. Kemp	Kew
10/1/1884	<i>The Argus</i>	9	1	Mr. Wm. Hurst	South Melbourne
10/1/1884	<i>The Argus</i>	9	1	Mr. Wm. Foster	Brunswick
10/1/1884	<i>The Argus</i>	9	1	Mr. D. Gotard	
4/11/1884	<i>The Argus</i>	9	1	Mr. F. T. Conway	Yea
2/12/1884	<i>The Age</i>	5	1	Mr. S. Merriman	South Melbourne
8/1/1885	<i>The Argus</i>	5	1	Mr. Charles Coles	St Kilda
8/1/1885	<i>The Argus</i>	5	1	Mr. Malpas	Mount Ridley, Craigieburn
8/1/1885	<i>The Argus</i>	5	1	Mr. A. H. S. Lucas	
20/5/1885	<i>The Age</i>	6	1	Mr. Spurr	Fitzroy
21/8/1885	<i>The Age</i>	7	1	Mr. John McMahon	Trafalgar, Gippsland
8/10/1885	<i>The Argus</i>	7	1	Mr. Wm. Bott	William St, Melbourne
8/10/1885	<i>The Argus</i>	7	1	Mr. Hugh Gilmour	Broadmeadows
3/12/1885	<i>The Argus</i>	10	1	Mr. A. H. Olsson	Macedon
3/12/1885	<i>The Argus</i>	10	1	President of the Shire of Echuca, Rochester	
22/12/1885	<i>The Argus</i>	5	1	Mr. William Davies jun.	Coburg
22/12/1885	<i>The Argus</i>	5	1	Dr. H. R. Bell	
4/3/1886	<i>The Argus</i>	11	1	Mr. J. Fleming	Park St, Brunswick
21/4/1886	<i>The Age</i>	6	1	Mr. A. Howre	Hotham
24/6/1886	<i>The Argus</i>	9	1	Mr. Shaw	Coranderrk Aboriginal Station
8/7/1886	<i>The Argus</i>	9	1	Miss C. Steward	Church St, North Fitzroy
19/10/1886	<i>The Argus</i>	10	1	Master Joseph Barrett	South Melbourne
19/10/1886	<i>The Argus</i>	10	1	Mr. James Webb	Gippsland
3/11/1886	<i>The Argus</i>	10	1	Mr. R. Sparks	
9/3/1887	<i>The Argus</i>	7	1	Mr. W. H. Pangbourne	Carlton

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Date	Paper	Page	No. koalas	Donated by	Of
7/9/1887	<i>The Argus</i>	6	1	Mr. F. W. Burne	William St, Collingwood
21/12/1887	<i>The Argus</i>	5	1	Mr. Henry Harris	Fitzroy
20/1/1888	<i>The Age</i>	6	1	Mr. Alfred Weaver	116 Queens Parade
20/1/1888	<i>The Age</i>	6	1	Mr. Harry Cox	Trafalgar
7/3/1888	<i>The Argus</i>	4	1	Mr. Skinner	Bank St, South Melbourne
7/3/1888	<i>The Argus</i>	4	1	Mr. H. Cox	Trafalgar
21/3/1888	<i>The Argus</i>	5	1	Freestone Brick Company	Brunswick
19/6/1888	<i>The Argus</i>	5	1	Mr. Marstin	Hotham
1/8/1888	<i>The Argus</i>	8	1	Mr. Hertie Kohry	Ellacombe, Newport
6/10/1888	<i>The Argus</i>	11	1	Messrs. A. Campbell & N. Morrison	Hamilton
6/10/1888	<i>The Argus</i>	11	1	Mr. J. H. Elliot	North Fitzroy
17/10/1888	<i>The Argus</i>	16	1	Mr. Lowe	Clifton Hill
31/10/1888	<i>The Age</i>	13	1	Master Thomas D. Parry	Drouin
20/2/1889	<i>The Argus</i>	11	1	Mr. W. J. Cruddas	Glenferrie Rd, Malvern
4/9/1889	<i>The Argus</i>	11	1 white	Mr. F. Laner	Drouin, South Gippsland
22/11/1889	<i>The Argus</i>	11	1	Mr. J. Burston	Carlton
22/11/1889	<i>The Argus</i>	11	1	Mr. Magreath	Fitzroy
9/7/1890	<i>The Argus</i>	5	1	Mr. M. J. Brennan	Melbourne
9/7/1890	<i>The Argus</i>	5	1	Mr. F. H. McCarthy	Charleville
3/9/1891	<i>The Argus</i>	11	1	Mr. Stuart	Windsor
17/8/1892	<i>The Age</i>	7	1	Mr. T. Brown	Keilor
6/12/1893	<i>The Age</i>	7	1	Mr. F. Marshall	Carlton
8/2/1894	<i>The Age</i>	3	2	Mr. W. Irvine	Burwood



Chapter 8 | foreword

Having established methods to non-invasively sample DNA from koalas, this project sought to investigate genetic structure and diversity in the South Gippsland koala population.

Survey of genetic diversity in the South Gippsland koala population is a high priority action included in *Victoria's Koala Management Strategy*¹. Previous genetic studies showed that the South Gippsland koala population has greater genetic diversity than koalas of French Island origin. However, no significant difference between koalas from South Gippsland and Phillip Island have been shown using nuclear markers. It was therefore also considered important to determine whether the South Gippsland koala population was differentiated from koalas of Phillip Island origin to unequivocally confirm that the South Gippsland population is a true remnant population.

To investigate genetic structure and diversity in the region we obtained as many samples as possible across South Gippsland. In conjunction with Friends of the Strzelecki Koala (South Gippsland Landcare), we conducted several workshops where project information and scat collection kits were provided to interested members of the community, who later provided samples from areas that would otherwise have been extremely difficult to sample (e.g. private agricultural land on the Gippsland Plain).

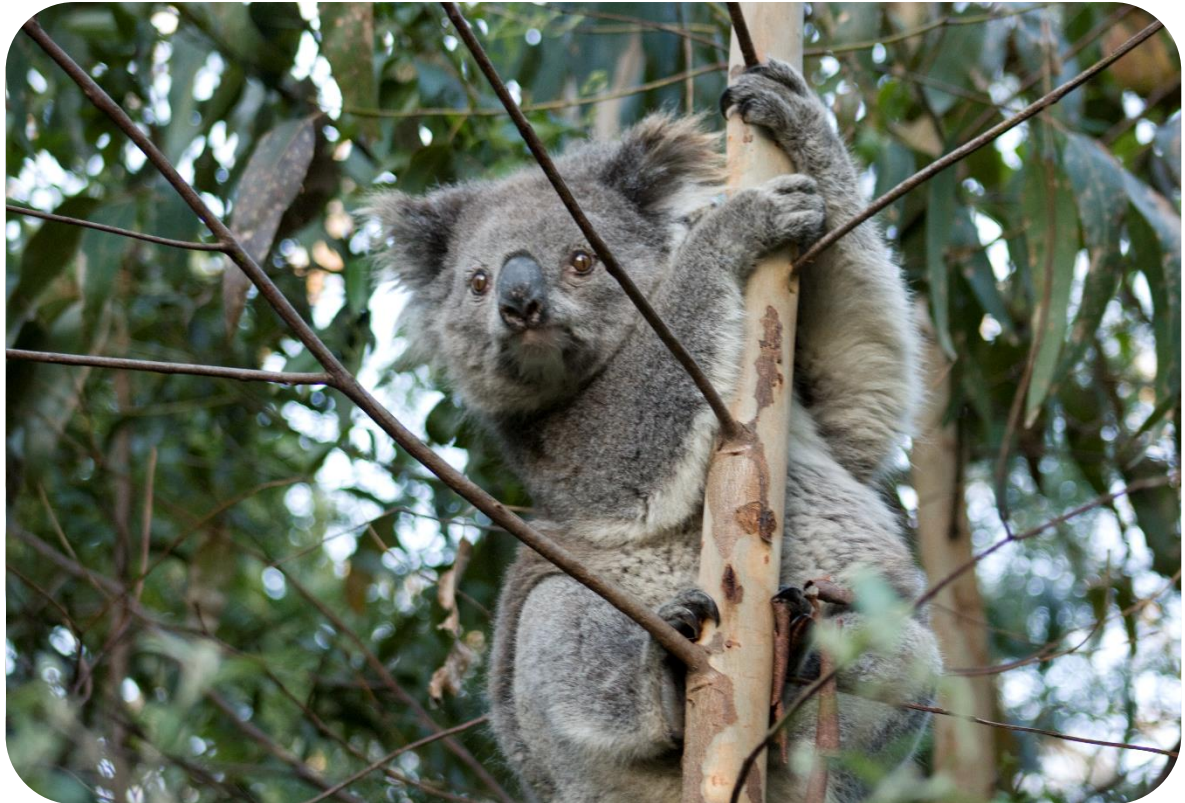
Scats were collected from a total of 661 koalas for this project. DNA was isolated from all submitted samples and screened for DNA quantity and quality before undertaking further analyses. Chapter eight demonstrates that the South Gippsland koala population has greater genetic diversity than other southern koala populations tested, and is genetically differentiated from other southern and northern reference populations. Due to its genetic distinctiveness, it is suggested that the remnant koala population in South Gippsland should be a conservation priority.

¹ Menkhorst P (2004) *Victoria's koala management strategy* Victorian Government Department of Sustainability and Environment, East Melbourne.



Chapter 8

Genetic structure and diversity of the koala population in South Gippsland, Victoria: a remnant population of high conservation significance



Submitted to *Conservation Genetics*



Chapter 8

Genetic structure and diversity of the koala population in South Gippsland, Victoria: a remnant population of high conservation significance

Abstract

In the Australian state of Victoria, the history of koalas and their management has resulted in the homogenisation and reduction of genetic diversity in many contemporary populations. Decreased genetic diversity may reduce a species' ability to adapt to future environmental pressures such as climate change or disease. The South Gippsland koala population is considered to be unique in Victoria, as it is believed to be a remnant population, not originating from managed populations that have low genetic variation.

This study investigated genetic structure and diversity of koalas in South Gippsland, with comparison to other populations in Victoria (French Island/Cape Otway, FI and Raymond Island, RI), New South Wales and south east Queensland. Population analyses were undertaken using both microsatellite genotype and mitochondrial DNA sequence data. Non-invasive sampling of koala scats was used to source koala DNA, allowing 222 South Gippsland koalas to be genotyped.

Using nuclear data the South Gippsland koala population was found to be significantly differentiated (D_{jost} 95% CI: SG–RI=0.03–0.06 and SG–FI=0.08–0.12) and more diverse (A_R 95% CI: SG=4.7–5.6, RI=3.1–3.3, FI=3.0–3.3; $p=0.001$) than other Victorian koala populations, supporting the premise that koalas in the South Gippsland region are part of a remnant population, not derived from translocated island stock. These results were also supported by mitochondrial data where eight haplotypes (Pc4, Pc17, Pc26, Pc27, and Pc56–Pc59) were identified in South Gippsland while a single haplotype (Pc27) was found in all

island koalas tested. Compared to other Victorian koala populations, greater genetic diversity found in South Gippsland koalas, may provide this population with a greater chance of survival in the face of future environmental pressures. The South Gippsland koala population is, therefore, of high conservation significance, warranting the implementation of strategies to conserve this population and its diversity into the future.

Introduction

The koala (*Phascolarctos cinereus*) is an arboreal Australian marsupial inhabiting eucalypt forests of Australia's east (Fig. 1). A dietary specialist, koalas feed exclusively on the foliage of certain eucalypt species (Martin & Handasyde 1999). Breeding occurs from October to May and females generally bear one offspring each one to three years (Handasyde *et al.* 1990; Martin & Handasyde 1990). Mean home range size varies from 0.5 ha at Cape Otway in Victoria (Whisson *et al.* 2016) to 135 ha in central Queensland (Ellis *et al.* 2002), likely driven by the density of preferred eucalypt species in an area (Martin & Handasyde 1999).

In Australia, extensive habitat loss and hunting post European colonisation (~1788) decimated koala populations. By the early 1900s, less than 1,000 koalas remained on the Victorian mainland, whilst introduced populations on French and Phillip Islands flourished, eventually reaching unsustainable densities and requiring intervention (Lewis 1954; Menkhorst 2008). Between 1923 and 2006, over 12,000 koalas were translocated from French and Phillip Islands to the mainland, to curb population growth whilst simultaneously facilitating the re-establishment of koala populations in Victoria (Lewis 1954; Menkhorst 2008). As only small numbers of individuals were used to establish the island populations during the late 1800s (French Island, $n=3$ and Phillip Island, $n\sim 10-30$), genetic diversity was reduced in island populations relative to their ancestral population/s (Lee *et al.* 2011; Wedrowicz *et al.* 2017b). Although translocation of individuals from French and Phillip Islands to the mainland was successful in re-establishing koala populations throughout Victoria (and in establishing koala populations in South Australia), genetic diversity among and between contemporary koala populations in Victoria and South Australia is low (Houlden *et al.* 1996; Houlden *et al.* 1999; Cristescu *et al.* 2009; Lee *et al.* 2011). Low genetic diversity can impact a species' ability to adapt to new environmental pressures such as climate change or disease, even where

population size is large (Bijlsma *et al.* 2000; Frankham 2005). This lack of variation is of genuine concern for the future viability of southern koala populations (in Victoria and South Australia), especially during the current period of rapid environmental change.

Koala populations in Victoria and South Australia are currently considered secure, mainly due to high koala densities of some populations (Department of the Environment 2015). The density of koalas in other southern populations ranges from low to moderate though data are unavailable for many (EaCRC 2011a). Conversely, widespread decline of koala populations in the north of Australia (Queensland, New South Wales and the Australian Capital Territory) since the 1990s has resulted in northern koalas being listed as vulnerable under the *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act; Department of the Environment 2015). Due to overabundance of some koala populations in Victoria and South Australia, koalas in these states are not listed as threatened under the EPBC Act (EaCRC 2012; Department of the Environment 2015).

As most koala populations in Victoria and South Australia were entirely founded by island stock they are likely to lack genetic diversity. An exception is the koala population in South Gippsland (Victoria), which is thought to be a remnant population that has received very few translocations of island stock (mainly in coastal South Gippsland; see Wedrowicz *et al.* 2017b). Koalas inhabiting this region may, therefore, retain greater levels of ancestral diversity; past studies have indicated that koalas in South Gippsland have greater genetic diversity compared to southern populations founded by island stock (Houlden *et al.* 1999; Lee *et al.* 2011).

The koala population in South Gippsland has been shown to be differentiated from and have significantly higher levels of genetic diversity (with an average of six alleles per locus) compared to koala populations from the French Island and Mornington Peninsula (of French

Island origin, with an average of less than four alleles per locus) (Lee *et al.* 2011). Genetic differences between the South Gippsland and Phillip Island populations have been demonstrated using mitochondrial DNA haplotype data with four haplotypes identified in South Gippsland and one at Phillip Island (Houlden *et al.* 1999). Using nuclear DNA however, the Phillip Island population was found to have similar levels of genetic diversity to the South Gippsland population (Houlden *et al.* 1996; Fowler *et al.* 1998).

Past population genetic studies of koalas in South Gippsland have largely relied on spatially localised sample collection or opportunistic sampling of deceased (e.g. road kill) individuals or animals entering wildlife shelters due to illness or trauma (Houlden *et al.* 1996; Houlden *et al.* 1999; Lee *et al.* 2011). Wild individuals in the Strzelecki Ranges bioregion (within South Gippsland, Fig. 1) have not been systematically sampled in the past, so the full extent and distribution of the genetic diversity within the regions koala population is unknown.

While molecular techniques are now routinely used for wildlife studies, sourcing DNA can be challenging. DNA for genetic studies is often obtained invasively from blood or biopsies sourced from captured or deceased animals. Although providing high quality DNA, invasive sampling has the potential to limit sample size, especially if the species is elusive and/or rare. Hence, obtaining sufficient samples to avoid bias is not always possible. Collection of DNA from non-invasive sources, such as scats, can be a more appropriate sampling option for rare or elusive species in difficult environments (Piggott & Taylor 2003). Sourcing DNA from scats reduces the time, cost and expertise associated with invasive sampling, and can thus facilitate the collection of more samples, in a shorter period of time, across a larger spatial area. As koalas spend most of their time in the canopy of tall eucalyptus trees (often >30 metres), animal capture for DNA sampling is difficult. Within the Strzelecki Ranges bioregion, obtaining DNA from wild koalas is especially difficult due to the rugged terrain.

Faecal pellets (scats) found at the base of a tree provide an alternative, accessible DNA source. Koala DNA isolated from host cells coating the surface of koala scats has been shown to provide DNA of quality sufficient to generate a unique identifying genotype (Wedrowicz *et al.* 2013).

DNA isolated from koala scats was used to investigate genetic variation within the South Gippsland koala population, using microsatellite genotyping and mitochondrial sequencing. The aims of this study were 1) to examine genetic differentiation and diversity of the South Gippsland koala population compared to other Victorian koala populations and more northern populations in New South Wales and Queensland and 2) to consider fine-scale population structure and the distribution of genetic variability in koalas throughout the South Gippsland region.

Methods

South Gippsland study site

The South Gippsland study region covers an area of approximately 6,000 sq km and includes the Strzelecki Ranges and Gippsland Plain bioregions¹ (Fig. 1). The Gippsland Plain bioregion is dominated by agricultural land, although relatively large forested areas exist in the east, consisting mainly of plains grassy forest, lowland forest and heathy woodland EVCs². Land use in the Strzelecki Ranges bioregion is more diverse. The western half of the Strzelecki Ranges bioregion mainly consists of agricultural land, with very few parks and reserves. In the eastern half of the Strzelecki Ranges bioregion, a large proportion of the landscape is under the management of Grand Ridge Plantations Pty. Ltd. (HVP Plantations)

¹ Bioregions are areas of land defined by similarities in geological and ecological characteristics; and are used by state government agencies, and others, for biodiversity planning and land management purposes.

² Ecological vegetation classes (EVCs) are used for classifying vegetation types within bioregions.

and is utilised for forestry. HVP's estate consists of plantation species, as well as native forest managed for conservation purposes (EaCRC 2011b). Native habitat containing preferred tree species, and therefore koalas, are unevenly distributed across the region. Within the HVP estate, koalas are found at densities of 0.25 koalas per hectare in native forest containing locally preferred eucalypt species including blue gum (*Eucalyptus globulus*), yellow stringybark (*E. muelleriana*) and/or mountain grey gum (*E. cypellocarpa*) (Allen 2015; Richard Appleton, HVP, pers. comm.). Plantation species in the region include radiata pine (*Pinus radiata*), blue gum, mountain ash (*E. regnans*) and shining gum (*E. nitens*). Koalas are generally less common within the plantations (R. Appleton, HVP, pers. comm.).

Sample collection

Koala scats were collected across South Gippsland between March 2013 and December 2016 by researchers, forestry staff, contractors and citizen scientists (Fig. 1). As koala density is greatest in the eastern part of the Strzelecki bioregion, an intensive sampling strategy was designed; the region was divided into nine areas, and searches for koalas and koala scats were carried out at between five to ten sites within each area. Areas considered to be good koala habitat (according to koala habitat modelling undertaken by HVP) were preferentially searched over less favourable habitat. Additionally, scat samples were opportunistically obtained from koalas at the Southern Ash Wildlife Shelter (SAWS), where sick and injured koalas from the South Gippsland region are rehabilitated. Tissue samples, from deceased individuals, were also obtained from SAWS.

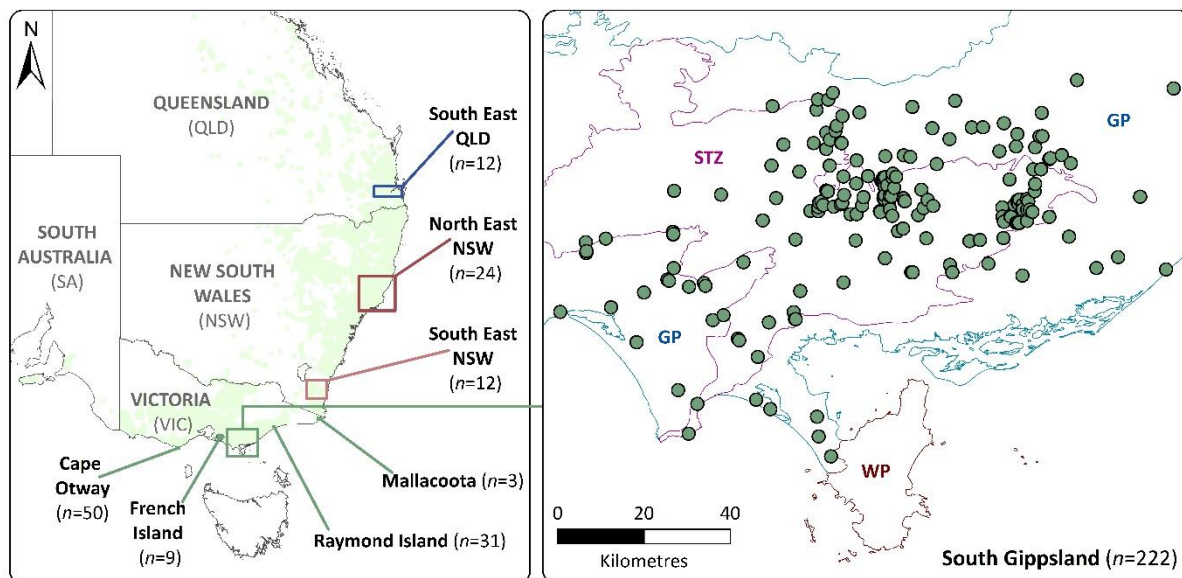


Figure 1 Spatial distribution of samples collected for this study. Shading on the map of eastern Australia (left) shows koala distribution which was adapted from Department of the Environment (2015). The map on the right shows the South Gippsland region, with the Strzelecki Ranges (STZ) bioregion indicated by the purple outline, the Gippsland Plain (GP) bioregion outlined in blue and the Wilsons Promontory (WP) bioregion outlined in red.

Seven reference populations were sampled for comparison; four from Victoria and three from New South Wales and Queensland. All reference populations were sampled by collecting scats. Victorian populations sampled included French Island, two mainland locations where individuals from French Island were released (Cape Otway and Mallacoota) and Raymond Island, which was founded by koalas translocated from Phillip Island. More northern populations sampled were from New South Wales (south east and north east) and south east Queensland (Fig.1). Prior information regarding potential sub structuring within populations was not known. A list of samples used for this study and their locations is provided in the supporting information (Table S1).

For all wild koalas, and some shelter koalas, four scats per individual (where possible) were collected using a wooden toothpick inserted into the side of each pellet. Scats were stored by inserting the opposite end of the toothpick into a foam block. Foam blocks supporting scats on toothpicks were then encased in a plastic, open ended covering (rectangular drain pipe sectioned into approximately 13 cm lengths) for protection. Each set of scats, stored in this way, constituted a sample from one individual. Details of each sample were recorded on the plastic covering, including date, collector details and spatial coordinates obtained from a hand held Global Positioning System (GPS). For deceased shelter animals, tissue samples were collected by SAWS by excising a small ($\sim 10 \times 20$ mm) piece of ear tissue and storing in methylated spirits. If possible, the location (nearest town) from which the deceased individual koala had been retrieved was georeferenced to obtain spatial data.

DNA isolation from scats

Scats were stored at ambient temperature, on toothpicks until surface washing (< 4 weeks). The surface of each scat was individually washed in a vial, with 2 mL of PBS buffer, by rolling on a Ratek roller mixer (BTR5P) at full speed for 8 mins. DNA isolation was carried out either immediately after surface washing or after storage of surface washes at -20°C (for a maximum of 10 months). DNA was isolated from two washes using the Qiagen QIAamp[®] DNA Stool Mini Kit as previously described (Wedrowicz *et al.* 2013) or the Axygen[®] AxyPrep[™] MAG Soil, Stool, and Water DNA Kit. Isolations using the Axygen[®] DNA Kit were carried out following the manufacturer's instructions with slight modification to the volumes of supernatant transferred after the centrifugation step (400 μL), SBW buffer added to the supernatant (400 μL) and binding enhancer (15 μL). DNA was isolated from two of the four washes to provide two separate DNA isolates per individual. To minimise the risk of cross contamination between samples, surface washing, DNA isolation, PCR setup and

electrophoresis of PCR products were all carried out in separate work areas using equipment dedicated to each work space and filter pipette tips were used. DNA was isolated from tissue using the DNeasy[®] Blood & Tissue Kit (Qiagen) following the manufacturer's protocol.

Screening for DNA quantity and quality

DNA isolates from two washes from each sample were screened for DNA quantity and quality as described in Wedrowicz *et al.* (2017a). Total DNA was quantified using the Qubit[®] dsDNA HS assay kit (Life Technologies) while DNA quality was assessed by amplification of microsatellite Pcv31 (Cristescu *et al.* 2009) and sexing markers using standard PCR and electrophoresis. Primers, IMY1 and IMY2 (Watson *et al.* 1998), used to amplify Y chromosome DNA in male koalas sampled in Victoria and New South Wales, did not produce amplification product for koalas sampled from Queensland (Fig. 1). A new primer set targeting the Y chromosome was therefore designed from GenBank sequence LC111530.1 (Katsura *et al.* 2016). Primers designated PCY-F (5'-TCTGGAGAATCCCAAATGC-3') and PCY-R (5'-ATTCTTCCCTGTGTTTAGCG-3') successfully amplified a fragment of approximately 130 base pairs in length for male Queensland koalas. For each sample, the DNA isolate producing the brightest bands using gel electrophoresis was chosen for microsatellite genotyping. DNA isolates that failed both screening PCRs were not analysed using microsatellite genotyping but were retained for potential amplification of mitochondrial DNA.

Microsatellite genotyping

Twelve microsatellite markers, K2.1, K10.1, Pcv2, Pcv6.1, Pcv6.3, Pcv24.2, Pcv25.2, Pcv30, Pcv31 (Cristescu *et al.* 2009), Phc2, Phc4 and Phc13 (Houlden *et al.* 1996) were used to genotype samples. Amplification and product separation using capillary electrophoresis were conducted at the Australian Genome Research Facility (AGRF), Melbourne, Australia.

Genotypes were replicated three or four times according to total DNA concentration of the sample (Wedrowicz *et al.* 2013).

DNA binning and the production of consensus genotypes were undertaken using R statistical software (R Core Team 2014). Raw microsatellite allele sizes were visualised and binned using the *MsatAllele* package (Alberto 2009). *ConGenR* (Lonsinger & Waits 2015) was used to generate consensus genotypes from replicate data, which were then checked by eye.

Genotypes with less than eight successfully amplified and scored loci were removed.

Allelematch (Galpern *et al.* 2012) was used to identify identical or almost identical genotypes (pairs with less than three mismatched loci, potentially representing matching genotypes with errors) as per Paetkau (2003). Identical genotypes, and genotypes with a small number of mismatched loci that could not be refuted as errors, were removed from the dataset.

Genetic statistics

The R package *strataG* (Archer *et al.* 2016) was used to test for deviations from Hardy-Weinberg (HW) proportions and to calculate the number of private alleles (A_P) for each population. Observed (H_O) and expected (H_E) heterozygosity, allelic richness (A_R) and the proportion of total sampled alleles found in each population ($A_{\%}$) were calculated using the *diveRsity* package (Keenan *et al.* 2013). The *corPlot* function in *diveRsity* plots differentiation against locus polymorphism to investigate potential bias in F_{ST} type estimates (Keenan 2014). It was found that F_{ST} was likely to be biased for this data, in which case D_{jost} (Jost 2008) is suggested as a more suitable measure of genetic differentiation (Keenan 2014), although it is recommended that other measures of differentiation be used in combination with F_{ST} (Meirmans & Hedrick 2011). Both F_{ST} and D_{jost} were therefore used to estimate genetic differentiation. Genetic and geographic distances were calculated and Mantel tests conducted in the R package *adeigenet*.

Population structure

Analyses for the detection of population structure were carried out using the microsatellite genotype data and the Bayesian clustering programs, STRUCTURE 2.3.4 (Pritchard *et al.* 2000) and BAPS 6.0 (Corander *et al.* 2008). Both STRUCTURE and BAPS group individuals into clusters in such a way that deviations from HW proportions and linkage disequilibrium (LD) are minimised, but differ in the way that the number of populations (K) is inferred (Latch *et al.* 2006). Spatial Bayesian clustering methods in GENELAND (Guillot *et al.* 2008) were used for the analysis of the fine scale genetic data. Population structure was also analysed using discriminant analysis of principal components (DAPC) in *adegenet*, which is based on genetic distances rather than minimisation of HW proportions and LD (Jombart 2008).

The STRUCTURE software was run with admixture and correlated allele frequencies using 3,000,000 Markov chain Monte Carlo (MCMC) iterations preceded by a burn-in of 1,000,000 iterations for K from 1–20. The most likely number of clusters inferred by STRUCTURE was chosen based on both Pritchard *et al.* (2010), where the most likely K has the lowest posterior probability from high values that have plateaued, and ΔK described by Evanno *et al.* (2005), which is based on the rate of change between log probabilities for consecutive values of K. BAPS was run ten times each using the non-spatial model for maximum values of K between 5 and 30 (5–15, 20, 25, 30). The most likely value of K was chosen based on the 10 partitions with the lowest marginal likelihoods (Corander *et al.* 2013).

Excluding samples without reliable spatial location data (i.e. those sampled from the shelter), GENELAND was used to test for fine scale genetic substructure in South Gippsland.

Population data were analysed in GENELAND using a spatial model and correlated allele frequencies. The maximum number of populations, K, was set to 20 with 1,000,000 iterations

and an additional 50,000 burn-in iterations. The thinning parameter was set to 1,000 and 20 independent runs were carried out. As recommended by Guillot (2012), the most likely number of clusters inferred by GENELAND and/or the best model amongst runs was chosen according to the run with the highest average posterior probability. Convergence was assessed by seeking evidence of non-convergence as described in the GENELAND manual (Guillot 2012).

Landscape data

ArcGIS 10.0 (Esri 2010) was used to investigate differences in habitat types between population clusters (inferred by GENELAND) within South Gippsland. Ecological Vegetation Classification (EVC) data for public land were obtained from DELWP (2016), while data for EVCs and dominant tree species within plantation estate were provided by HVP. Data from DELWP and HVP were merged to provide a single layer containing vegetation information for both public (DELWP) and privately (HVP) managed land across the region (at a cell size of 25 m × 25 m). Cluster assignment data for individuals, inferred by GENELAND, were then overlaid onto the vegetation layer. Using the join function in ArcGIS, individuals were then assigned to a habitat type, based on their sampling location.

Mitochondrial sequence data

Three regions of the mitochondrial genome (mtDNA) were targeted for sequencing, including approximately 700 bp of the mitochondrial control region (Fowler *et al.* 2000), 1001 bp of the cytochrome B gene (*cytB*) and a 1559 bp stretch of DNA spanning genetic sequence of NADH dehydrogenase subunits five and six (*ND5/6*). Primers for the amplification of *cytB* and *ND5/6* were designed using koala mitochondrial sequence (Genbank accession NC_00813: Munemasa *et al.* 2006) and Primer-BLAST (Ye *et al.* 2012). The control region PCR used primers KmtL1 and KmtH2 designed by Fowler *et al.* (2000); *cytB* DNA was

amplified using primers *cytB*-F (5'-CCCATCCAACATCTCTACCT-3') and *cytB*-R (5'-ATGTGGTGGATGCTACTTGG-3') and the *ND5/6* PCR used primers, *ND*-F (5'-CGCAACAGGAAAATCAGCCC-3') and *ND*-R (5'-TAGTTAGTGGTGGCTTGGGG-3').

Mitochondrial PCRs were carried out using BIO-X-ACT™ Short DNA Polymerase (Bioline) or MyTaq™ 2X Red Mix (Bioline). Reactions carried out with BIO-X-ACT™ Short DNA Polymerase consisted of 1 X OptiBuffer, 0.25 X Hi-Spec additive, 2 mM MgCl₂, 0.5 mM each dNTP, 0.25 µM of each primer and 1 unit of BIO-X-ACT™ Short DNA Polymerase made up to 20 µL with water. PCRs using MyTaq™ Red Mix were made up using 0.25 µM of each primer, 0.1 µg/µL bovine serum albumin (BSA) and 1X MyTaq™ Red Mix made up to a total volume of 40 µL with water.

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequencing was carried out at AGRF, Melbourne. Where a haplotype was observed only once, PCR and sequencing were repeated independently to confirm. Sequences were trimmed and aligned using MEGA 6 (Tamura *et al.* 2013). Aligned sequences were exported in FASTA format for use by the R packages, *apex* (Jombart *et al.* 2017), *pegas* (Paradis 2010) and *ape* (Paradis *et al.* 2004), in which sequences were concatenated and a haplotype network produced. Estimates of nucleotide diversity (π), haplotype diversity (h) and pairwise differences between populations were obtained using ARLEQUIN 3.5.2.2 (Excoffier & Lischer 2010) while haplotype AMOVA and differentiation (Φ_{ST}) were calculated using GenAlEx 6.5 (Peakall & Smouse 2012).

Results

Sampling and microsatellite genotyping

Scats were collected from a total of 583 putative individuals during this study. DNA quality was high with DNA samples from 467 (80%) putative individuals passing the quality screening. After removal of genotypes with less than eight (out of 12) successfully amplified and scored loci, 429 (74%) samples provided reliable genotypic data. Matching genotypes indicated that 67 individuals had been sampled more than once. After removing duplicates, 362 individual koalas had been sampled. The majority of the individuals sampled ($n=222$, 61%) were from the South Gippsland (SG) region; 188 were obtained from scats and 34 from ear tissue. Genotypes from scat samples were obtained from both wild ($n=155$) and shelter ($n=33$) koalas in the SG region. Genotypic data were also obtained from reference populations in Victoria (total $n=93$); Cape Otway (OTW, $n=50$), French Island (FI, $n=9$), Mallacoota (MC, $n=3$) and Raymond Island (RI, $n=31$) and from interstate populations (total $n=48$); south east New South Wales (SENSW, $n=12$), north east New South Wales (NENSW, $n=24$) and south east Queensland (SEQLD, $n=12$).

Using MICROCHECKER, there was no evidence for null alleles within the microsatellite loci, except for Pcv2 in the SG and OTW populations. Genotypes were in HW proportions for all loci and populations, except for Pcv2 for the SG, RI and OTW populations and Pcv25.2 for the FI group. The Pcv2 locus was retained, as the spatial distribution of Pcv2 genotypes in the SG and OTW populations identified regions where homozygotes for offending alleles were clustered, suggesting population structure.

Population structure

Using STRUCTURE, the ΔK method described by Evanno *et al.* (2005) indicated $K=2$ as the most likely number of population clusters, which divided Victorian samples from more northern (SENSW, NENSW and SEQLD) koala populations with very high cluster membership; 96% of northern individuals and 100% of southern individuals had a q value higher than 0.90. The ΔK method of inferring the number of population clusters can, however, suffer from falsely inflated values at $K=2$ (Campana *et al.* 2011), making it important to also analyse other relevant values of K for biological meaning. The ΔK plot showed an additional, less intense, peak at $K=7$ and the maximum log probability of the data ($\text{LnP}(D)$) also indicated the most likely number of populations to be $K=7$. The seven clusters inferred by STRUCTURE also indicated differentiation between Victorian and more northern koala populations, however, Victorian koalas were further divided into three main clusters, those of French Island origin (FI, OTW and MC), Phillip Island origin (RI) and South Gippsland (SG). Cluster assignment for individuals of French Island and Phillip Island origin were high, with 85% (52/61) and 74% (23/31), respectively, of individuals having cluster assignment (q) values greater than 0.8. One individual appearing to be of SG origin was detected on RI, suggesting it had been translocated. Further structure was also inferred in SG with four population clusters being present, however, a greater amount of admixture was found in SG evidenced by a lack of strong cluster membership (Fig. 2).

The population structure determined by BAPS correlated well with the STRUCTURE results, however, the number of clusters inferred by BAPS for the data set was double that of STRUCTURE, at $K=14$ (Fig. 2). As with STRUCTURE, individuals sampled in Victoria were clustered into six distinct groups, those of French Island origin (OTW/FI/MC), those of Phillip island origin (RI) and SG which was divided into four population sub clusters (Fig. 2).

BAPS distinguished between the three interstate sample locations where STRUCTURE did not. Within the more northern reference populations, BAPS divided individuals into clusters which matched their sampling location. The two sampling sites in SENSW were clearly separated, divided according to coastal or inland sampling regions. Individuals sampled from Queensland were also separated into two groups, broadly corresponding to individuals sampled in coastal or more inland regions. Discriminant analysis of principal components (DAPC) in *adegenet* by sampling location supported population structuring provided by BAPS (Fig. S1). Further population structuring in the SG koala population was also indicated using DAPC, where five sub clusters were inferred (Fig. S2).

Fine scale genetic structure in South Gippsland

STRUCTURE, BAPS and DAPC all gave an indication of further population structure in SG, supported by F_{ST} values ranging from 0.03 to 0.06 between the four BAPS assigned populations in SG. Fine scale population structure in SG was therefore investigated using GENELAND, where seven population clusters were inferred. Six spatially well-defined population clusters with more than five assigned individuals were identified (Fig. 3). Population structure in some regions was not well defined, with individuals from multiple population clusters present.

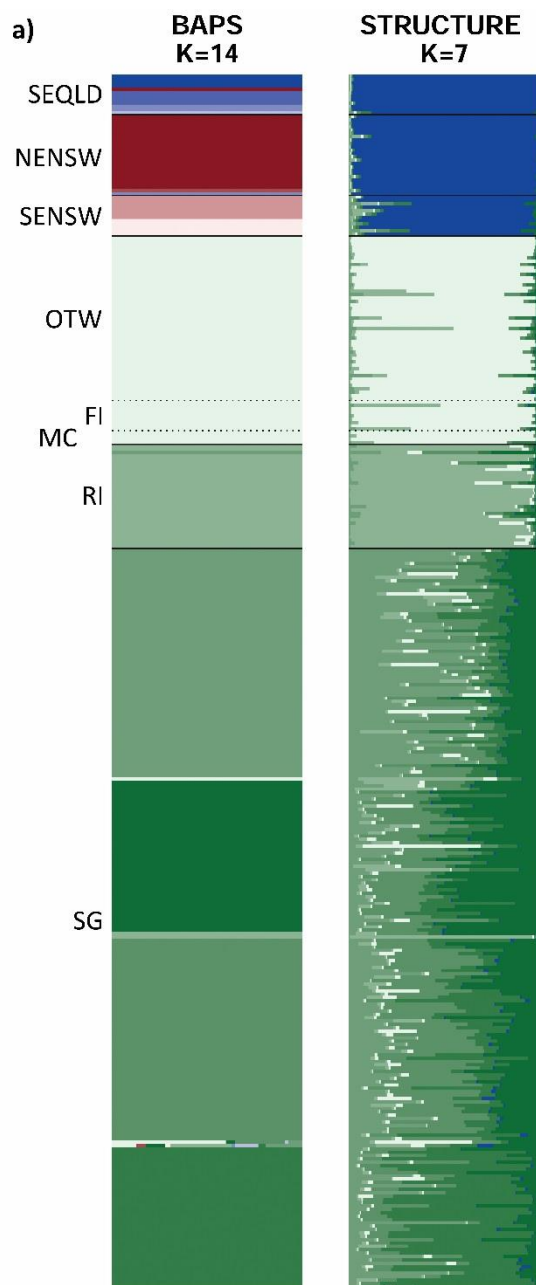
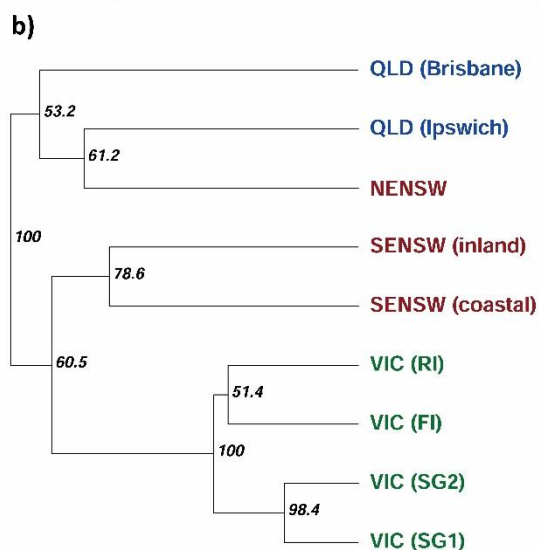


Figure 2 a) Inferred population structure using STRUCTURE and BAPS. Horizontal bar plots represent 362 individual koalas. Different colours on the same horizontal bar represent the estimated proportion of the individual's ancestry assigned to a particular population cluster. Solid black lines separate different sample locations while dotted black lines separate samples from Cape Otway, French Island and Mallacoota (all French Island descent). Sample areas are labelled **SEQLD** South east Queensland ($n=12$), **NENSW** North east New South Wales ($n=24$), **SENSW** South east New South Wales ($n=12$), **OTW** Cape Otway ($n=50$), **FI** French Island ($n=9$), **MC** Mallacoota ($n=3$), **RI** Raymond Island ($n=31$) and **SG** South Gippsland ($n=222$)

b) Neighbour joining tree using genotypic data and Provesti's distance based on the main population clusters identified using BAPS. Clusters with less than three individuals were excluded while the four clusters identified in the South Gippsland population were simplified to two by combining clusters with F_{ST} values less than 0.04.



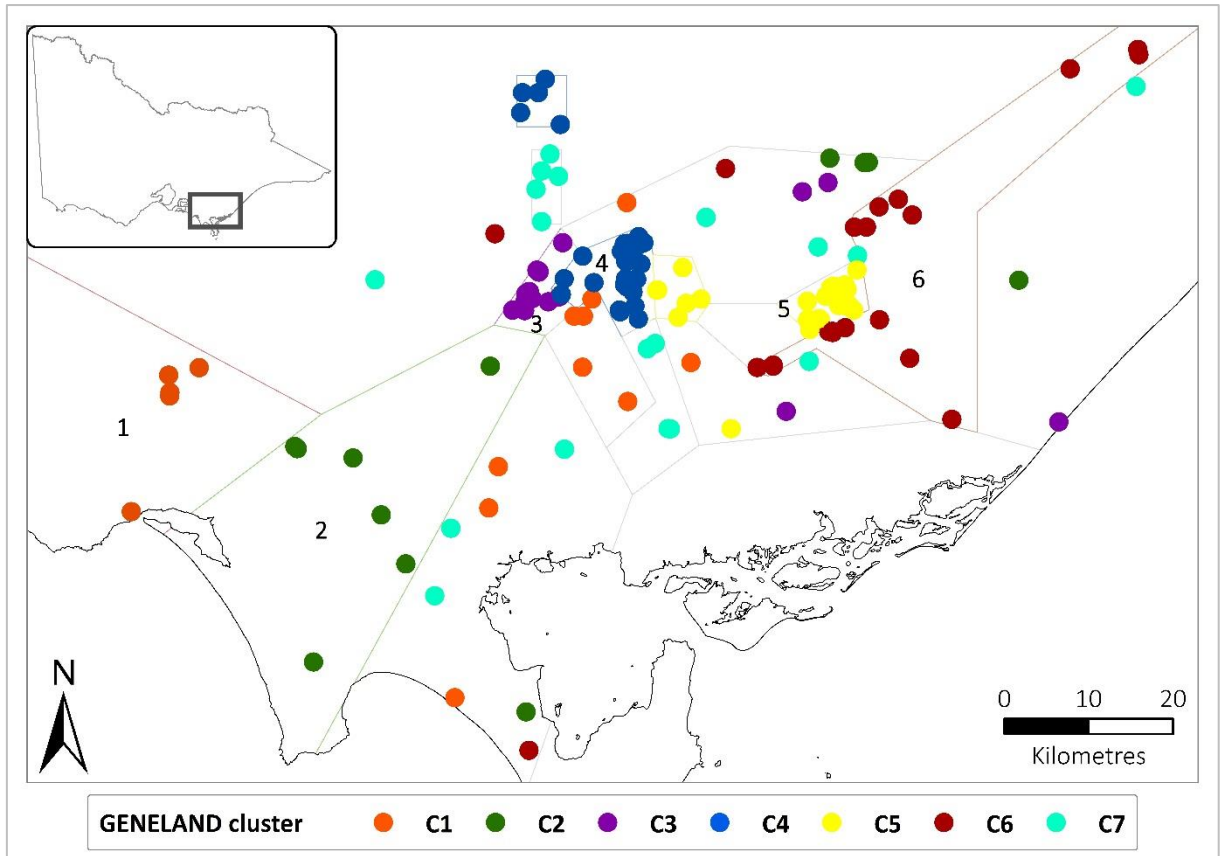


Figure 3 Population substructure in South Gippsland inferred using GENELAND ($n=165$). Each point represents a sampled individual and colours are indicative of different population clusters, which are overlayed with polygons delineating regions of population clustering for greater clarity. Spatially well-defined population clusters with more than six individuals are numbered 1 through to 6.

All six population clusters were significantly differentiated using D_{jost} , but five of the fifteen comparisons were not significant using F_{ST} (Table S2). Genetic differentiation between SG population clusters ranged from 0.0002 (cluster 2 – cluster 3) to 0.09 (cluster 1 – cluster 5) for D_{jost} and between 0.003 (cluster 1 – cluster 3) to 0.12 (cluster 1 – cluster 5) for F_{ST} . Genotypes conformed to HW proportions for all six populations at all loci except for locus Pcv6.1 in population cluster 4, potentially indicative of further fine scale structure. Genotypic AMOVA between Victorian populations (SG, RI and FI) indicated 9% between population variation,

4% variation between the six subpopulations in SG and 0.4% variation between individuals within subpopulations.

Using GIS, the spatial distribution of three (clusters 3, 4, and 5) of the seven population clusters inferred by GENELAND (Fig. 3) appeared to correspond to the distribution of differing habitat types (Fig. S3 and Fig. S4). Cluster 3 was located at a site that consisted mainly of lowland forest (consisting of messmate and peppermint) and, to a lesser extent, damp forest (where messmate, blue gum and mountain grey gum are the common tree species). Koalas in cluster 4, were mainly found in native forest within HVP estate, where blue gum is the dominant species. On public land, koalas within cluster 4 were mostly sampled within herb-rich foothill forest and damp forest (consisting of messmate, blue gum and mountain grey gum). Lastly, koalas in cluster 5 were concentrated where yellow stringybark is the dominant tree species.

Fine-scale isolation by distance

Discrete population structure can arise due to the presence of clinal structure (isolation by distance, IBD) and conversely, the detection of clinal structure can result from the presence of discrete structure (Meirmans 2012). Determining whether population structure is discrete or clinal can therefore be difficult (Ruiz-Gonzalez *et al.* 2015). A Mantel test between all wild sampled individuals in SG indicated the presence of IBD ($p=0.002$). Tests for IBD within GENELAND inferred populations were, however, not significant ($p>0.23$) except for population cluster 4 ($p=0.008$). Detection limits may, however, be affected by small sample sizes. It is therefore unclear whether the population structure in SG is discrete, clinal or a mixture of the two.

Concatenated mtDNA haplotypes

Concatenated DNA sequence data (control region, *cytB* and *ND5/6*) were obtained for a subset of samples ($n=55$) representing populations from both Victorian (SG $n=15$, OTW $n=3$ and RI $n=6$) and more northern (QLD $n=9$, NENSW $n=9$, SENSW $n=13$) reference sites. After alignment and trimming of mitochondrial DNA sequences, 641 bp of the control region, 933 bp of *cytB* and 1381 bp of *ND5/6* were obtained. Sequence data were deposited in GenBank under accession numbers KY979201–KY979210 (control region), KY979211–KY979220 (*cytB*) and KY979221–KY979230 (*ND5/6*). Concatenated sequence consisting of 2955 DNA base pairs identified 20 haplotypes across sampling areas (Fig. 4a). Apart from Hap16, which was found in both SG and in French and Phillip Island derived populations, all haplotypes were specific to a given region that was sampled. Compared to the control region alone, inclusion of the *cytB* (933 bp) and *ND5/6* (1381 bp) regions provided an additional 26 variable sites which were able to differentiate individuals with identical control region haplotypes present in separate regions (Table S3). For example, koalas sampled around 350 km apart (from SG and coastal SENSW) were found to have the same control region haplotype, Pc17, but could be differentiated by a variable site in the *ND5/6* region. Sequence data for the *cytB* and *ND5/6* mtDNA regions did not add greatly to the discrimination of samples collected in the south, as different haplotypes detected for *cytB* (three Victorian haplotypes) and *ND5/6* (two Victorian haplotypes) were generally associated with a specific control region haplotype.

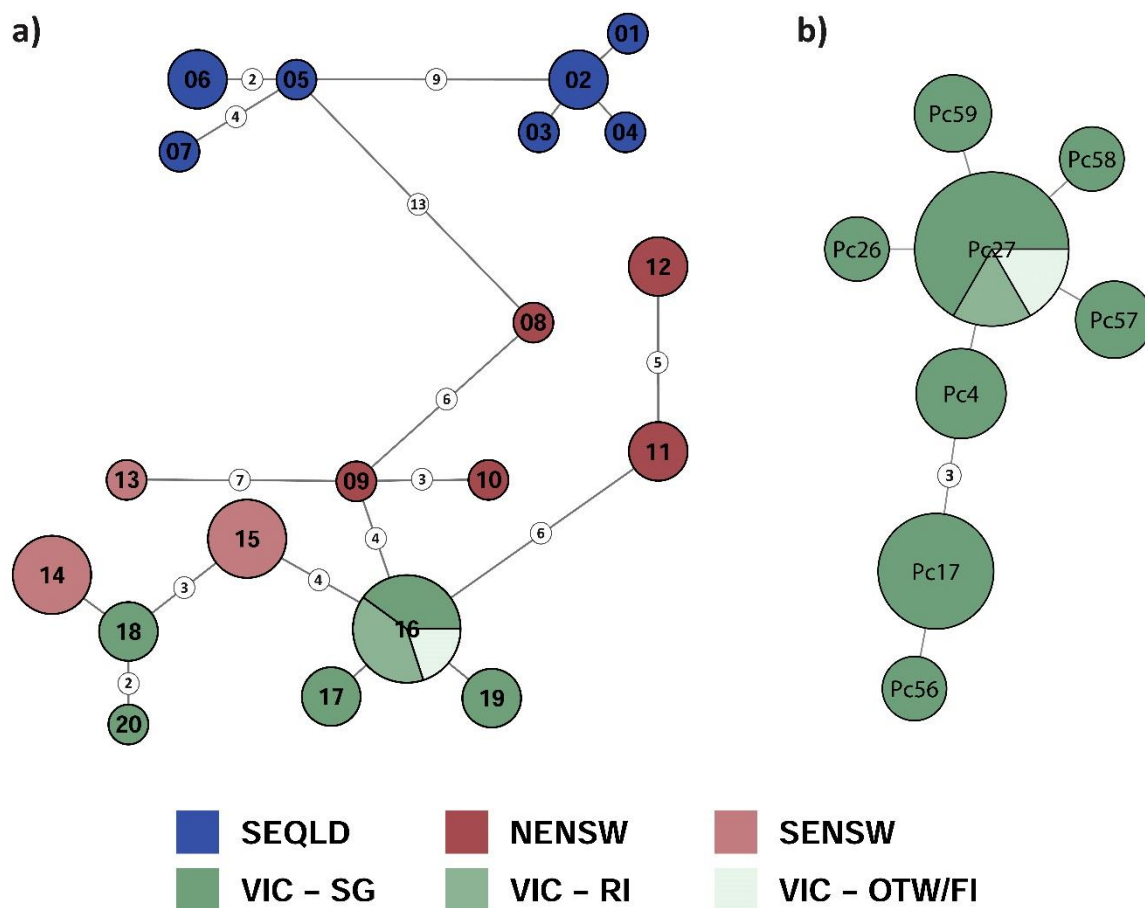


Figure 4 a) Haplotype network based on 2955 bp of concatenated mtDNA sequence (control region, *cytB* and *ND5/6*). Sampled populations were South Gippsland (SG, VIC), Raymond Island (RI, VIC), Cape Otway/French Island (OTW/FI, VIC), south east New South Wales (SENSW), north east New South Wales (NENSW) and south east Queensland (SEQLD). Haplotypes are numbered 01 to 20 which correspond to Hap01 to Hap20 in the text. The number of base pair differences between haplotypes are shown in small white circles on the lines adjoining haplotypes. Lines joining haplotypes differing by one base pair are unlabelled.

b) mtDNA control region haplotype network for Victorian samples.

Mitochondrial control region haplotypes

To obtain a large sample set for the investigation of haplotype diversity in the South Gippsland region, mtDNA was sequenced at the control region alone for an additional 150 randomly selected samples from Victoria (SG, $n=110$, OTW/FI, $n=20$, RI, $n=20$). Six previously unreported mtDNA control region haplotypes were detected in this study; four in the SG study area (Pc56, Pc57, Pc58 and Pc59) and one each in SENSW (Pc55) and NENSW (Pc54) (detected in samples for which concatenated mtDNA sequences were obtained). New control region haplotypes were named using standardised labels as recommended by Neaves *et al.* (2016). Relationships between control region haplotypes detected in Victorian koalas sampled are illustrated by the haplotype network in Fig. 4b. The control region haplotype network was star shaped with the dominant haplotype (Pc27) surrounded by five low frequency variants (Pc4, Pc26, Pc57, Pc58, Pc59), suggestive of recent expansion in evolutionary terms (Fig. 4b). Two slightly more divergent control region haplotypes, Pc17 and Pc56 were also found in SG (Fig. 4b). Control region haplotypes supported the population clusters identified by GENELAND, with differences in the haplotype frequencies detected in each population cluster (Table 1).

Table 1 Genetic statistics for the six population clusters (with six or more individuals) in South Gippsland inferred by GENELAND (Fig. 3) using microsatellite genotypes (upper section) and mitochondrial control region sequence data (lower section). Haplotypes identified in only one population cluster are shown in bold.

Cluster	1	2	3	4	5	6
N	6	7	12	38	25	20
A	3.2	4.0	4.1	5.2	4.7	3.8
A_%	55	67	69	81	77	65
A_R	2.9	3.3	3.3	3.3	3.0	3.0
P_A	1	3	0	6	3	2
H_O	0.58	0.67	0.64	0.59	0.50	0.57
H_E	0.55	0.59	0.61	0.57	0.49	0.55
<i>n^{mt}</i>	5	22	7	14	13	13
<i>nh</i>	2	3	2	4	3	3
$\pi \pm sd$ %	0.06 \pm 0.08	0.11 \pm 0.10	0.09 \pm 0.09	0.21 \pm 0.10	0.12 \pm 0.10	0.31 \pm 0.21
Pc4	20%	27%	-	-	-	-
Pc17	-	5%	-	14%	8%	23%
Pc27	80%	68%	57%	71%	85%	69%
Pc56	-	-	-	-	-	8%
Pc57	-	-	-	7%	8%	-
Pc58	-	-	-	7%	-	-
Pc59	-	-	43%	-	-	-

n^{mt}: Number of sequences, **nh**: Number of haplotypes, **n^{pm}**: Number of polymorphic sites, **pw**: Average number of pairwise differences, **h \pm sd %**: Haplotype diversity, **$\pi \pm sd$ %**: Nucleotide diversity, **N**: Number of individual genotypes, **A**: Allelic diversity; the mean number of alleles per locus, **A_%**: The percentage of alleles, from all populations, found in each specific population, **A_R**: Allelic richness; the mean number of alleles per locus, corrected for differences in sample size (based on a sample size of six) **P_A**: Private alleles; alleles unique to a single population, **H_O**: Observed heterozygosity, **H_E**: Expected heterozygosity.

Genetic differentiation between Victorian populations

Private alleles are unique to a particular population and can provide an indication of genetic distinctiveness. In SG, 38 alleles not found in other Victorian koala populations were detected, indicating that koalas in SG are genetically distinct from the island populations sampled. There was moderate differentiation between the SG and RI (D_{jost} 0.04 and F_{ST} 0.08) and SG and OTW/FI/MC (D_{jost} 0.10 and F_{ST} 0.12) populations.

Tests of differentiation (Φ_{ST}) using concatenated haplotypic data between the SG and island derived populations were not significant ($p=0.08$). Using control region haplotypes alone, for which a greater amount of data were available, significant differentiation between the SG and island derived populations was detected ($\Phi_{\text{ST}} = 0.07$, $p=0.04$), indicating that the SG population is also differentiated from populations of both French Island and Phillip Island origin at the mtDNA control region.

Broad scale genetic differentiation

For both genotypic and haplotypic data, genetic differentiation between populations increased with increasing distance (Table S4, Fig. S5). Using genotypic data, pairwise population differentiation was highly correlated to geographic distance (D_{jost} : $R^2=62\%$, $p=0.0005$, F_{ST} : $R^2=44\%$, $p=0.007$). Haplotypic data also indicated a pattern of isolation by distance (Average pairwise differences: $R^2=40\%$, $p=0.02$) between populations (Fig. S5).

When genotypic data were stratified as per the dendrogram in Fig. 2a, AMOVA showed between population variation of 16%, between subpopulation variation of 10% and within subpopulation variation of 3%. Variation in haplotypic data were more structured than the genotypic data, with 61% of haplotypic variation found between SEQLD and more southern

sample regions (NENSW, SENSW and VIC), alongside 20% between subpopulation variation and 19% within subpopulation variation.

Genetic diversity

The SG koala population was found to have greater genetic diversity than populations originating from French or Phillip Islands (OTW or RI, respectively). The SG koala population had a mean of 7.2 alleles per locus while the OTW and RI populations both had an average of 3.3 alleles per locus. Allelic richness (mean alleles per locus corrected for differences in sample size) was also found to be significantly greater in the SG population ($A_R=5.1$) compared to either of the island populations (OTW $A_R=3.2$; RI $A_R=3.3$, $F=9.2$, $p=0.001$) with an average of two extra alleles per locus (Table 2).

Using concatenated haplotypes, nucleotide diversity was higher in the SG (0.14 ± 0.007) population compared to the OTW/FI (0.00) and RI (0.00) populations which comprised a single haplotype (Table 2; Fig. 4a). Overall, eight Victorian control region haplotypes were identified (Fig. 4b). All eight were present in the SG koala population, while only one, the most common SG haplotype (Pc27), was identified in the island populations (OTW/FI and RI, $\chi^2=14.5$, $p=0.04$). These data indicate that the SG koala population is distinct and has significantly greater genetic diversity than other Victorian koala populations sampled.

The SG and SENSW koala populations had comparable allelic richness with respective averages of 4.2 and 4.1 alleles per locus (Table 2) while nucleotide diversity (concatenated mtDNA data) was slightly lower in the SENSW (0.11 ± 0.005) population compared to the SG population (0.14 ± 0.007). Compared to the SG population, higher nuclear genetic diversity was found in the NENSW and SEQLD populations, with allelic richness of 6.1 ($T=-2.3$, $p=0.04$) and 5.3 ($T=-3.4$, $p=0.006$) respectively (Table 2). Similarly, nucleotide diversity

using concatenated haplotypes was highest in the SEQLD (0.21 ± 0.015) and NENSW (0.20 ± 0.014) koala populations.

Table 2 Summary of genetic statistics for sampled populations using microsatellite genotypes (upper section) and concatenated mtDNA sequence (lower section). In the upper section, comparisons made between Victorian populations only are indicated by 'VIC' in superscript following the parameter label.

	VIC (SG)	VIC (OTW)	VIC (RI)	NSW (SE)	NSW (NE)	QLD (SE)
N	222	50	31	12	24	12
A	7.2	3.3	3.3	4.6	7.9	6.5
A%	63	32	32	43	69	56
A^{%VIC}	99	53	53	-	-	-
A_R	4.17	2.83	3.03	4.07	6.08	5.28
A_R^{VIC}	5.11	3.15	3.27	-	-	-
P_A	11	0	0	4	12	10
P_A^{VIC}	38	0	1	-	-	-
H_O	0.59	0.44	0.50	0.65	0.68	0.57
H_E	0.60	0.45	0.52	0.61	0.73	0.68
n^{mt}	15	3	6	13	9	9
Nh	5	1	1	3	5	7
n^{pm}	11	0	0	12	16	15
Pw	4.1	0	0	3.4	5.9	6.1
h ± sd %	73 ± 6	-	-	56 ± 14	74 ± 5	84 ± 2
π ± sd %	0.14 ± 0.007	-	-	0.11 ± 0.005	0.20 ± 0.014	0.21 ± 0.015

n^{mt}: Number of sequences, **nh**: Number of haplotypes, **n^{pm}**: Number of polymorphic sites, **pw**: Average number of pairwise differences, **h ± sd %**: Haplotype diversity, **π ± sd %**: Nucleotide diversity, **N**: Number of individual genotypes, **A**: Allelic diversity; the mean number of alleles per locus, **A%**: The percentage of alleles, from all populations, found in each specific population, **A_R**: Allelic richness; the mean number of alleles per locus, corrected for differences in sample size, **P_A**: Private alleles; alleles unique to a single population, **H_O**: Observed heterozygosity, **H_E**: Expected heterozygosity.

Discussion

The koala is an iconic species, endemic to Australia and is the last surviving member of the Phascolarctidae family. Low genetic variation is of genuine concern for the future viability of koala populations in Victoria and South Australia, as a lack of genetic diversity can affect population fitness, hindering the ability to adapt to future environmental change. In this study, we have shown that koalas in the South Gippsland region are differentiated from all other koala populations sampled. They also have a significantly greater level of genetic diversity compared to other Victorian koala populations, retaining a greater proportion of the ancestral diversity that was lost post European settlement. The importance of conserving the koala gene pool in the South Gippsland region therefore cannot be overstated, as they carry additional genetic diversity that is not present in populations established by island animals.

Conservation genetics can provide information that may alert managers to issues affecting a population's genetic health such as population isolation, limited gene flow and inbreeding. Obtaining sufficient, truly representative, sample sizes for genetic studies can, however, be difficult, slow and expensive. This study demonstrates the power of non-invasive sampling, using DNA from koala scats to obtain genetic data with the ability to inform and monitor conservation strategies. Probably one of the most concerning future environmental challenges for the koala, will be the effects of climate change (Ellis *et al.* 2010) which may alter koala habitat distribution and suitability (Adams-Hosking *et al.* 2012; González-Orozco *et al.* 2016) and modify leaf chemistries, potentially rendering currently preferred koala dietary species unsuitable (Moore & Foley 2000; DeGabriel *et al.* 2010). Molecular technologies, such as those described here, provide a tool for longitudinal genetic monitoring of population responses to environmental change. The rapid collection of contemporary, empirical data, will

expedite the acquisition of knowledge, allowing evidence based conservation strategies to be implemented and monitored over time.

South Gippsland koalas are genetically distinct from other Victorian populations

Genetic structure in populations occurs due to deviations from random mating which may result from differing levels of population isolation or fragmentation (Frankham *et al.* 2012). Population structure can help to reveal population ancestries, while the extent of differentiation between populations can provide a measure of how different two populations are; something that is particularly important to know when assessing risks associated with moving animals between populations for conservation purposes such as genetic rescue (Frankham *et al.* 2011; Frankham 2016).

As inferred by the STRUCTURE and BAPS plots (Fig. 2), the South Gippsland, Cape Otway (French Island origin) and Raymond Island (Phillip Island origin) populations were all moderately differentiated from one another (D_{jost} 0.04–0.10, F_{ST} 0.08–0.12), indicating that the South Gippsland koala population is distinct from koalas originating from both French and Phillip Islands. Previously reported levels of genetic differentiation between French Island derived populations and the South Gippsland population have ranged from weak (F_{ST} =0.05; Houlden *et al.* 1996) to moderate (F_{ST} =0.11; Seymour *et al.* 2001) to strong (F_{ST} =0.25; Lee *et al.* 2011).

In this wide-scale study, population substructure was evident within the South Gippsland region (Fig. 3; Table 1). Variable genetic differentiation estimates between studies may therefore be attributable to differing sampling locations, regimes and sizes. Discrepancies may also be due to the loss of particular subsets of the South Gippsland koala population due to events occurring between studies. For example, a high proportion of the samples used for the Lee *et al.* (2011) study were obtained from individuals who had succumbed to the 2009

bushfires; although searches were undertaken, few samples were obtained from the area affected by the 2009 bushfires for this study. It is therefore possible that different subsets of the diversity present in South Gippsland koalas have been sampled by different studies.

South Gippsland koalas have greater genetic diversity than other Victorian populations

Genetic variation is important as it provides populations with the capacity to adapt and survive environmental changes, while decreased variation is found to negatively affect survival, growth and reproduction rates (Reed & Frankham 2003; Frankham *et al.* 2012). Some koala populations founded by island stock are currently at a high density, presently appearing unaffected by their low diversity. These low diversity populations have, however, only existed for a relatively short time. Stochastic factors play a significant role in determining the outcome of low diversity for a population (Reed 2010). Currently overabundant koala populations may not yet have been subjected to pressures severe enough to cause widespread population decline and extirpation. Monitoring these populations for early signs of population decline may assist in their conservation should the negative effects of low genetic diversity become apparent in the future. Indeed, examples exist where koalas were once overabundant but have declined to extremely low densities or extirpation; these include Wilsons Promontory, Phillip Island and the Grampians National Park (Wedrowicz *et al.* 2017b).

Both genotypic and haplotypic data revealed a significantly greater level of genetic diversity in South Gippsland. These results indicate that the relatively small numbers of island koalas translocated to the area did not result in the swamping of local genetic diversity and translocated koalas may not have successfully integrated with resident populations at any level. It may be that the South Gippsland population had recovered to sufficient size by the

time these translocations occurred, such that low levels of integration would have had little effect on levels of differentiation and diversity.

Greater genetic diversity in the South Gippsland koala population could confer increased evolutionary potential relative to island derived populations in Victoria. However, although neutral genetic markers are commonly used to estimate evolutionary potential, the relationship may be weak (Reed & Frankham 2001); further work to directly estimate evolutionary potential using adaptive loci, in both South Gippsland and island derived populations, should be used to evaluate the risk of future declines due to low evolutionary potential.

Greater diversity in South Gippsland, compared to both Cape Otway (French Island origin) and Raymond Island (Phillip Island origin) populations, provides strong support that South Gippsland koalas are derived from remnant populations having survived in the region at a time when most other Victorian populations are thought to have become extirpated or reduced to extremely low numbers (Lewis 1934, 1954). This reinforces and extends studies conducted by Houlden *et al.* (1999) and Lee *et al.* (2011), which demonstrated genetic differences and greater diversity in the South Gippsland koala population compared to island derived populations (both French and Phillip Islands using mtDNA and French Island alone using nuclear DNA).

Population substructure is present in the South Gippsland koala population

Subtle population substructure within the South Gippsland koala population was detected using both genotype and haplotype data, although it is unclear whether the observed structure is discrete, clinal or a combination of both. In either case, the presence of genetic structure across the region indicates that gene flow is restricted. Further work is needed to investigate the reasons for population substructure in South Gippsland.

Predominant eucalypt species vary across discrete koala habitats within South Gippsland. Three population clusters identified by GENELAND correspond with differences between dominant tree species within the region occupied by each inferred cluster. Previous studies have shown high levels of site fidelity in koalas, demonstrating a strong tendency for philopatry (Mitchell 1990; Whisson *et al.* 2016). This may indicate a preference for individuals to remain in areas containing habitat similar to their natal area (Stamps & Swaisgood 2007), suggesting that the population substructure observed may reflect, in part, recent patterns of koala dispersal.

Another possibility is that the koala population in South Gippsland was continuous pre-European settlement but, as the forests were cleared for agriculture and the koala population dwindled, small numbers of individuals survived within isolated patches of habitat. When mass farm failures and abandonment occurred in early 1900s, leading to reforestation and conversion of much of the land to plantation (Legg 1986; Wedrowicz *et al.* 2017b), re-expansion of koala populations across the landscape may have resulted in the fine scale pattern of genetic structure observed here, where each cluster represents a koala colony isolated during the period of severe forest fragmentation.

Past and continuing levels of habitat fragmentation are also likely to have influenced patterns of genetic structure in South Gippsland. Further analyses using landscape genetic approaches (Storfer *et al.* 2006) would be useful to identify potential barriers to koala gene flow and gain insights into how koalas utilise differing landscapes within South Gippsland. Landscape genetic methods may also provide a greater understanding of the nature of the genetic structure detected in South Gippsland (Ruiz-Gonzalez *et al.* 2015).

Southern koala populations appear less diverse than northern populations

Isolation by distance occurs where gene flow between populations is sufficiently limited so as to result in the differentiation of neighbouring populations (Frankham *et al.* 2012). Both genotypic and haplotypic data showed a strong pattern of isolation by distance indicating that, historically, koalas (and their habitat) are likely to have been either continuously distributed (with limited dispersal) along Australia's east or consisting of a series of subpopulations for which low levels of migration could occur between adjacent populations (stepping stone model; Frankham *et al.* 2012). Due to local extinctions and habitat degradation, few koala populations are likely to remain connected by the low levels of gene flow that historically occurred across their range. This may have a negative effect on the conservation of genetic diversity for the species (Weeks *et al.* 2016).

Reconnecting nearby patches of koala habitat via corridors or stepping stones would be one strategy that could increase gene flow towards historic levels, thereby minimising further losses of genetic diversity. Reconnecting habitat will also be important because one response of wild populations to climatic changes may be to shift to their distribution to more suitable habitat (Nuñez *et al.* 2013; McGuire *et al.* 2016), something that may not be possible where habitats are separated by large distances.

Compared to koala populations in Victoria and South Australia, genetic diversity tends to be higher in the more northern populations (Houlden *et al.* 1996; Houlden *et al.* 1999). Koala populations are likely to have undergone substantial losses of genetic diversity Australia wide due to dramatic declines post European settlement. A number of European species, however, exhibit decreasing diversity in the direction of post glacial population expansion (Hewitt 1999). During the cold, dry conditions of the glacial periods, potential koala habitat and therefore koala populations may have been restricted to refugia in Queensland and/or north

east New South Wales (Adams-Hosking *et al.* 2011). More favourable climatic conditions in the preceding interglacial period may have allowed population expansion, with each subsequent founding event resulting in reduced genetic diversity in populations as they expanded southwards (Hewitt 1999). Lower genetic diversity in southern koala populations, such as South Gippsland and south east New South Wales, relative to more northern koala populations may thus be due, in part, to the koala's evolutionary history.

Genetic diversity present in the South Gippsland koala population must be conserved

Genetic diversity provides populations the ability to tolerate environmental changes, with the risk of extinction expected to be higher where genetic diversity is low (Frankham 2005; Frankham *et al.* 2012). Climates vary across the koala's range, as do genetic and morphological (Briscoe *et al.* 2015) characteristics of koalas. How koalas in any one region will respond to climatic and habitat changes is thus difficult to know. Conserving diversity across the entire range of the koala is therefore important.

Conclusions

The South Gippsland koala population is a remnant Victorian population, not derived from the koala translocation program. It has the highest known level of genetic diversity of all koala populations in Victoria and South Australia. Consequently, conservation of the South Gippsland koala population and its genetic diversity into the future is of high importance. The South Gippsland koala population requires a different management approach compared to other Victorian koala populations (where the focus is on the management of overpopulation), with an emphasis on conservation of this population and its genetic diversity. Due to high population densities in several southern koala populations, koalas in Victoria and South Australia were excluded from the 2012 EPBC listing of the koala as Vulnerable (EaCRC 2011a, b, 2012); the South Gippsland koala population ought to be an exception to that exclusion.

Koala management in Victoria is currently concentrated on preventing the devastating effects of overpopulation (Menkhorst 2008; DELWP 2015). As discussed, however, the lack of genetic diversity in high density populations may increase their chance of future declines. Other remnant koala populations (outside of South Gippsland) have not been identified in Victoria to date. Population remnants may be at low density, so an ability to carry out analyses from scat samples will greatly facilitate further investigation. Further widespread genetic surveys, in Gippsland and across Victoria, may highlight additional populations of conservation priority and inform strategies to minimise further losses of genetic diversity in southern koala populations.

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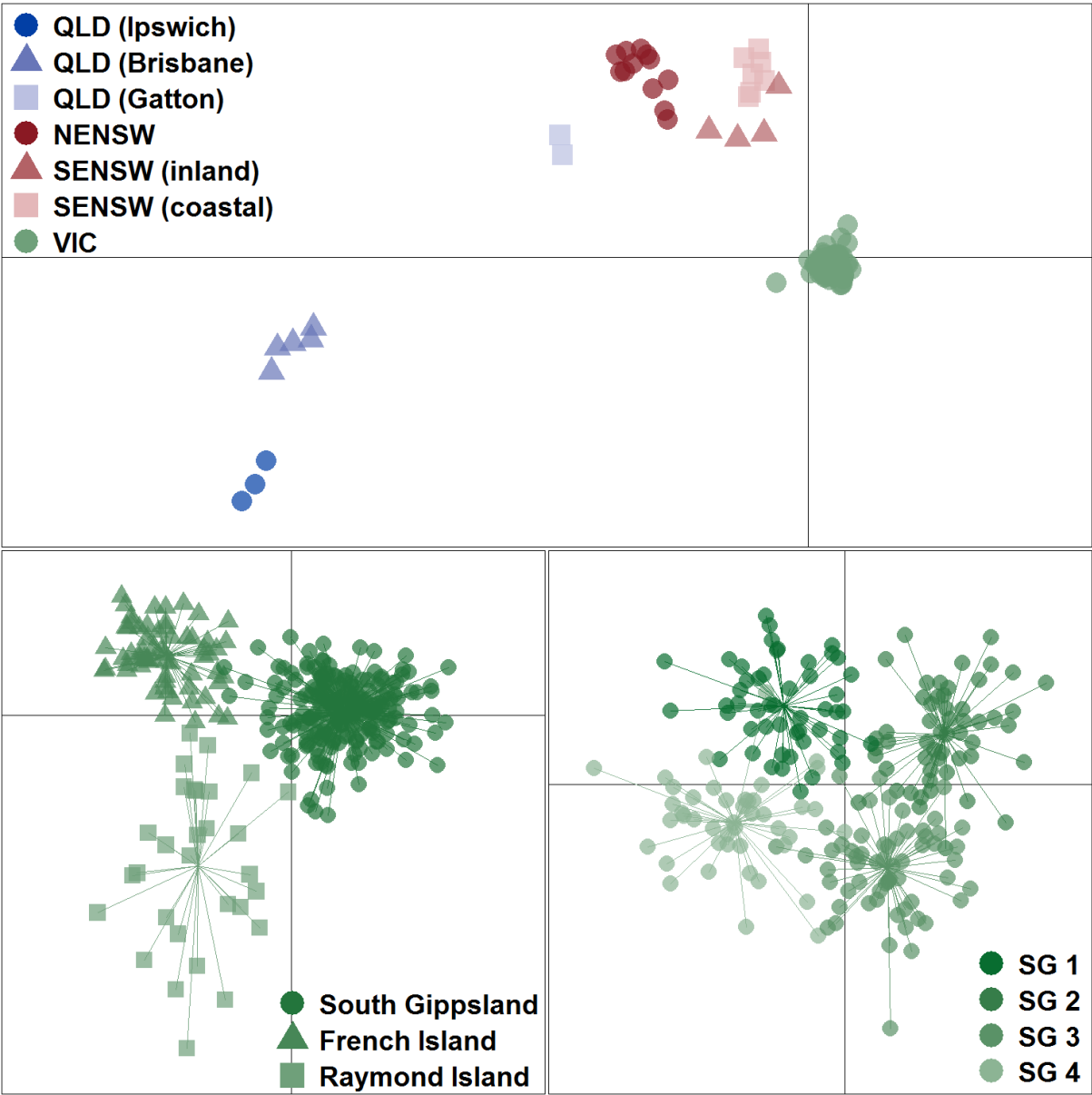


Figure S1 Australia wide (top) and Victorian (bottom left) population clustering according to sampling location using discriminant analysis of principal components (DAPC) in the *adeigenet* package. A DAPC scatterplot of individuals in South Gippsland according to population clusters inferred by BAPS (SG1–SG4) is shown at the bottom right.

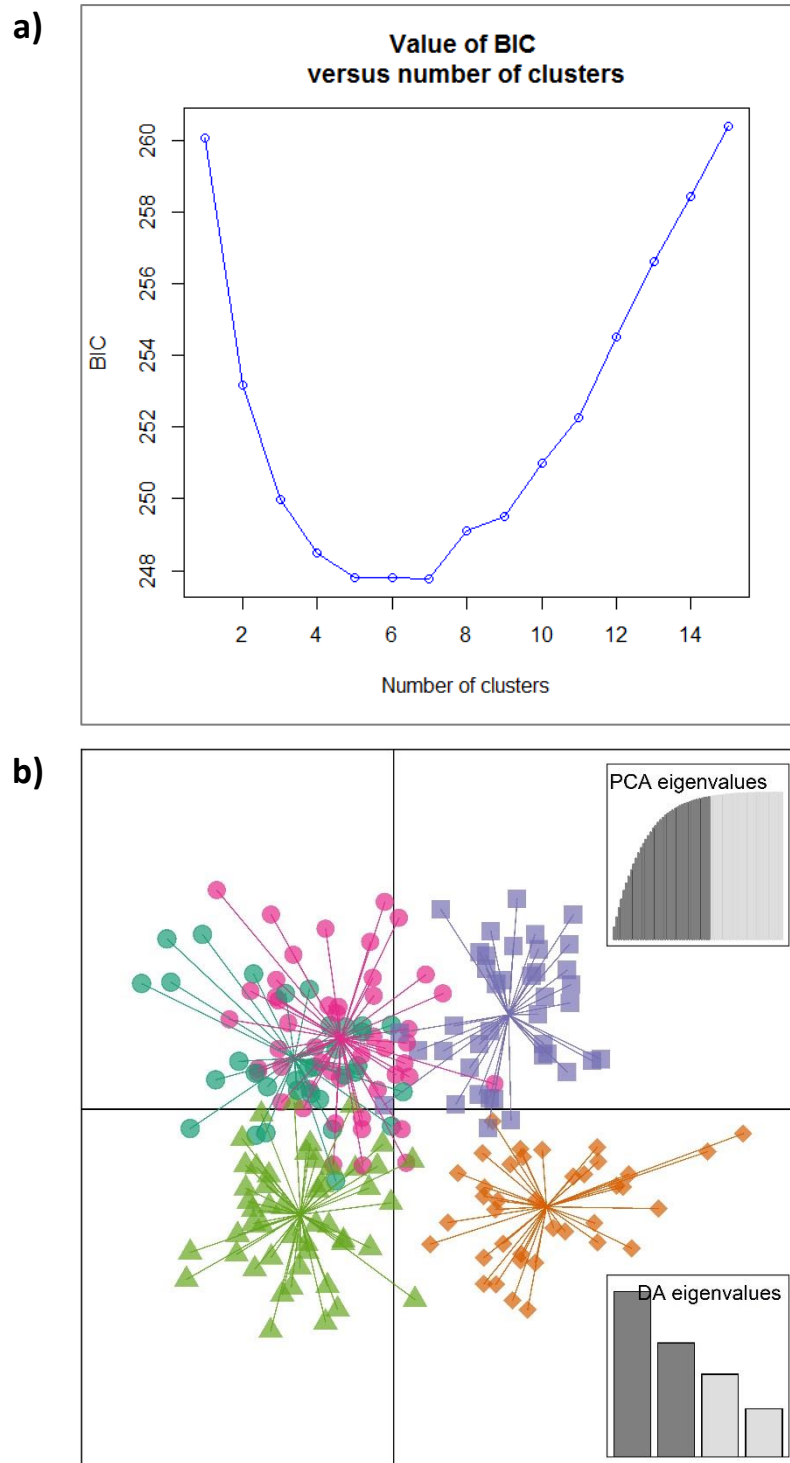


Figure S2 a) Five genetic clusters were identified in South Gippsland samples the DAPC find.clusters function in *adeigenet* where the number of clusters was indicated by the minimum BIC value (Jombart, 2008). **b)** DAPC scatterplot for South Gippsland samples using the five clusters inferred by *adeigenet* in part a).

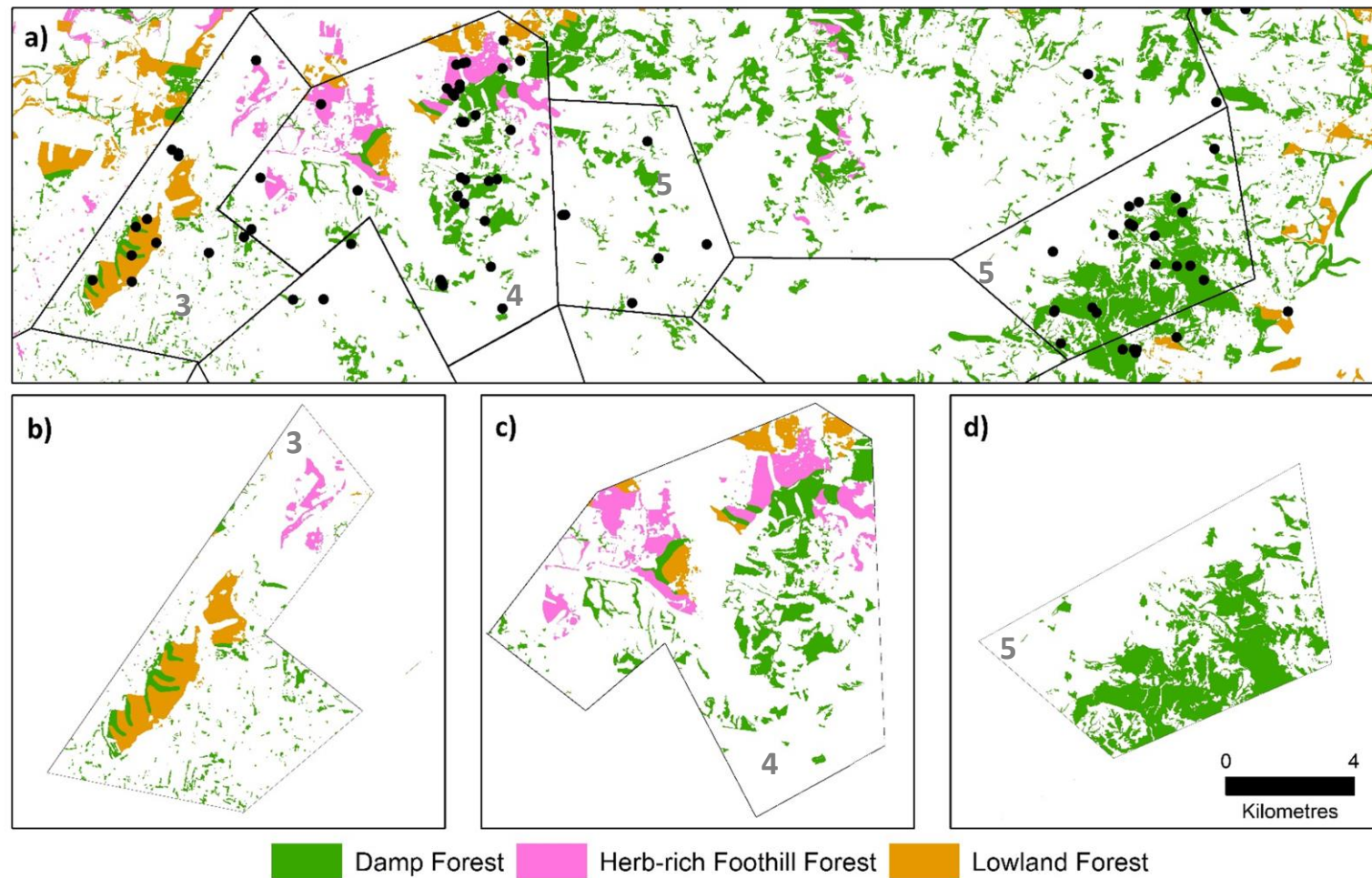


Figure S3 Differences in major ecological vegetation classes (EVCs) between GENELAND clusters 3, 4 and 5 (as shown in Fig. 3). Panel **a**) shows EVCs and koalas sampled (black dots) across the area corresponding to GENELAND clusters 3, 4 and 5. Panel **b**) shows EVCs in the area covered by cluster 3 where the main eucalypts are messmate, peppermint and mountain grey gum; panel **c**) shows EVCs in the area pertaining to cluster 4 where blue gum, messmate and mountain grey gum are common while **d**) shows EVCs for the eastern most area occupied by 'cluster 5' individuals, where yellow stringybark, mountain grey gum, messmate and blue gum are present.

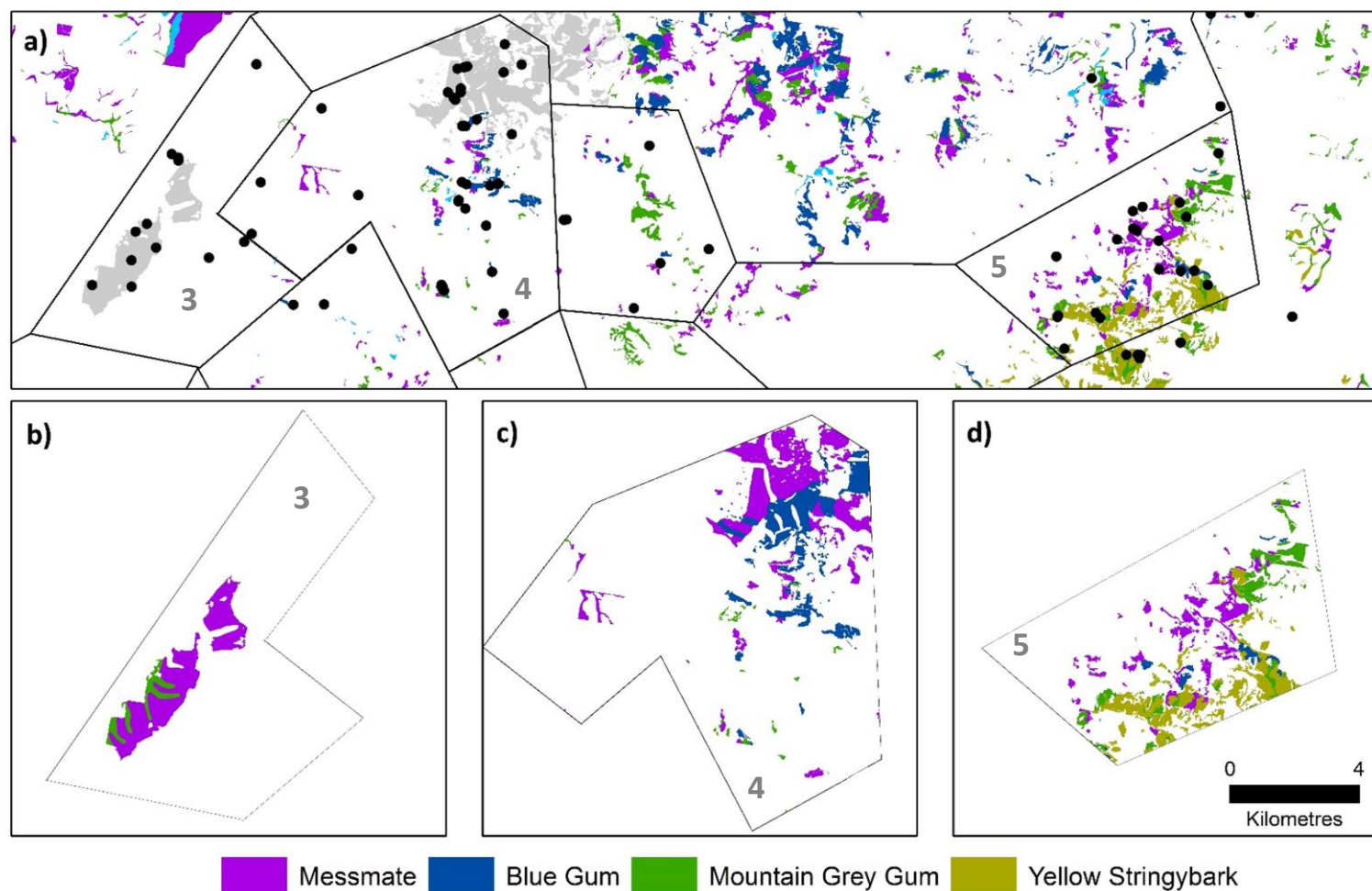


Figure S4 Differences in dominant eucalypt species between GENELAND clusters 3, 4 and 5 (as shown in Fig. 3). Panel **a)** shows dominant eucalypt species and koalas sampled (black dots) across the area corresponding to GENELAND clusters 3, 4 and 5. Grey shading indicates regions where data for dominant species were unavailable and EVC data were used to infer dominant species. Panel **b)** shows dominant eucalypt species in the area covered by cluster 3.

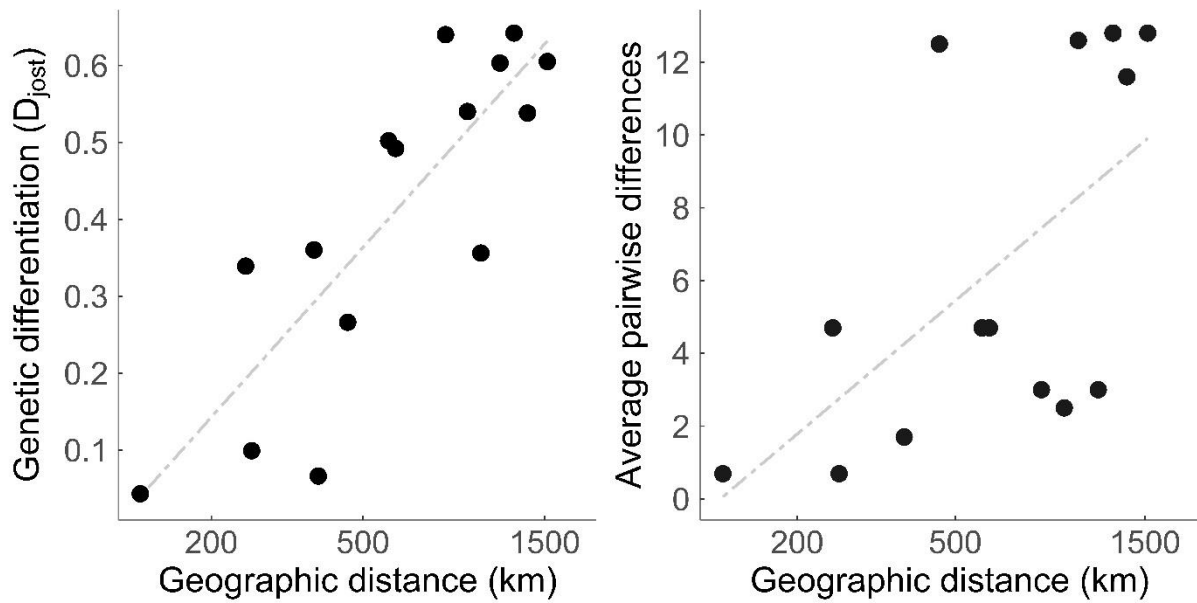


Figure S5 Relationship between pairwise genotypic genetic differentiation (D_{jost} using genotypic data and average number of pairwise differences in mtDNA sequence) between populations in table 1 (QLD, NENSW, SENSW, SG, FI and RI) and the base ten logarithm of geographic distance. Regression line equations were $D_{jost} = 0.00037 \log_{10}(\text{Geographic distance}) + 0.13$ ($R^2=62\%$, $p=0.0005$) and Concatenated mtDNA pairwise differences = $0.006 \log_{10}(\text{Geographic distance}) + 0.96$ ($R^2=40\%$, $p=0.02$).

Table S1 List of individuals sampled for this study along with their origin, assignment to genetic clusters (using BAPS) and mtDNA haplotypes.

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
1	VIC	SG	Allambee	C10		
2	VIC	SG	Allambee East	C7	Pc17	18
3	VIC	SG	Allambee East	C2		
4	VIC	SG	Balloong	C2	Pc27	
5	VIC	SG	Binginwarri	C10	Pc27	
6	VIC	SG	Binginwarri	C2		
7	VIC	SG	Binginwarri	C2		
8	VIC	SG	Binginwarri	C1		
9	VIC	SG	Binginwarri	C1		
10	VIC	SG	Boodyarn	C10	Pc27	
11	VIC	SG	Boodyarn	C2	Pc27	
12	VIC	SG	Boodyarn	C2	Pc17	
13	VIC	SG	Booran	C6	Pc27	
14	VIC	SG	Bruthen		Pc27	
15	VIC	SG	Bruthen	C2	Pc17	
16	VIC	SG	Budgerie	C1		
17	VIC	SG	Budgerie	C1		
18	VIC	SG	Budgerie	C6		
19	VIC	SG	Budgerie	C6		
20	VIC	SG	Budgerie	C1		
21	VIC	SG	Budgerie	C1		
22	VIC	SG	Budgerie	C1	Pc27	
23	VIC	SG	Budgerie	C10		
24	VIC	SG	Budgerie	C2		
25	VIC	SG	Budgerie	C1		
26	VIC	SG	Budgerie	C6		
27	VIC	SG	Budgerie	C6		
28	VIC	SG	Budgerie	C6	Pc27	
29	VIC	SG	Budgerie	C6		
30	VIC	SG	Budgerie	C6	Pc27	
31	VIC	SG	Budgerie	C6		
32	VIC	SG	Budgerie	C6	Pc57	
33	VIC	SG	Budgerie	C2		
34	VIC	SG	Budgerie	C6		
35	VIC	SG	Budgerie	C1		
36	VIC	SG	Budgerie	C2		
37	VIC	SG	Budgerie	C6	Pc27	
38	VIC	SG	Budgerie	C6	Pc27	
39	VIC	SG	Bulga		Pc27	16
40	VIC	SG	Bulga	C2	Pc27	
41	VIC	SG	Bulga	C2		
42	VIC	SG	Bulga	C1	Pc27	
43	VIC	SG	Bulga	C2		
44	VIC	SG	Bulga	C2		
45	VIC	SG	Bulga	C2		
46	VIC	SG	Bulga	C2		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
47	VIC	SG	Callignee	C6		
48	VIC	SG	Callignee	C2		
49	VIC	SG	Callignee	C1		
50	VIC	SG	Callignee	C2		
51	VIC	SG	Callignee	C6	Pc27	
52	VIC	SG	Callignee	C2		
53	VIC	SG	Callignee	C2		
54	VIC	SG	Callignee	C1		
55	VIC	SG	Callignee	C2		
56	VIC	SG	Carrajung	C1	Pc27	
57	VIC	SG	Carrajung	C1		
58	VIC	SG	Carrajung	C1	Pc27	
59	VIC	SG	Carrajung	C10	Pc27	
60	VIC	SG	Carrajung	C2		
61	VIC	SG	Carrajung	C1		
62	VIC	SG	Coolungoolun	C1	Pc27	
63	VIC	SG	Devon	C6	Pc27	
64	VIC	SG	Devon	C1		
65	VIC	SG	Doomburrim	C1		
66	VIC	SG	Doomburrim	C1		
67	VIC	SG	Doomburrim	C1		
68	VIC	SG	Drumdlemara	C6	Pc27	
69	VIC	SG	Drumdlemara	C10	Pc27	
70	VIC	SG	Dumbalk	C10	Pc27	
71	VIC	SG	Goon Nure	C1		
72	VIC	SG	Goon Nure	Pc17		
73	VIC	SG	Gunyah Gunyah	C6	Pc27	
74	VIC	SG	Hazelwood	C6		
75	VIC	SG	Jeeralang	C6	Pc57	17
76	VIC	SG	Jeeralang	C2		
77	VIC	SG	Jeeralang	C1		
78	VIC	SG	Jeeralang	C10		
79	VIC	SG	Jumbuk	C1		
80	VIC	SG	Jumbuk	C10		
81	VIC	SG	Jumbuk	C6	Pc27	
82	VIC	SG	Jumbuk	C6		
83	VIC	SG	Jumbuk	C2	Pc27	
84	VIC	SG	Jumbuk	C6	Pc27	
85	VIC	SG	Jumbuk	C2		
86	VIC	SG	Jumbuk	C6		
87	VIC	SG	Jumbuk	C6	Pc27	
88	VIC	SG	Jumbuk	C1		
89	VIC	SG	Jumbuk	C6	Pc27	
90	VIC	SG	Jumbunna East	C1		
91	VIC	SG	Jumbunna East	C13		
92	VIC	SG	Jumbunna East	C10		
93	VIC	SG	Kirrak	C6	Pc4	
94	VIC	SG	Knockwood	C10		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
95	VIC	SG	Kongwak	C1	Pc27	
96	VIC	SG	Kongwak	C10	Pc27	
97	VIC	SG	Kongwak		Pc27	
98	VIC	SG	Koorooman	C10		
99	VIC	SG	Leongatha	C10	Pc4	
100	VIC	SG	Leongatha	Pc4		
101	VIC	SG	Leongatha	Pc27		
102	VIC	SG	Leongatha	Pc27		
103	VIC	SG	Leongatha	C10		
104	VIC	SG	Leongatha	C2	Pc17	
105	VIC	SG	Leongatha	C10		
106	VIC	SG	Leongatha	Pc4		
107	VIC	SG	Leongatha	C10		
108	VIC	SG	Leongatha	C10	Pc27	
109	VIC	SG	Leongatha	C10	Pc27	
110	VIC	SG	Longford	C2	Pc27	
111	VIC	SG	Longford	C10	Pc56	20
112	VIC	SG	Longford	C6	Pc27	
113	VIC	SG	Longford	C1		
114	VIC	SG	Longford	C10		
115	VIC	SG	Longford	C6		
116	VIC	SG	Loy Yang	C1		
117	VIC	SG	Loy Yang	C6		
118	VIC	SG	Loy Yang	C6		
119	VIC	SG	Mardan	C1		
120	VIC	SG	Mardan	C10	Pc17	18
121	VIC	SG	Mardan	C6		
122	VIC	SG	Maryvale	C1		
123	VIC	SG	Meeniyah	C2	Pc27	
124	VIC	SG	Meeniyah	C1	Pc27	
125	VIC	SG	Mirboo	C6	Pc59	19
126	VIC	SG	Mirboo	C6		
127	VIC	SG	Mirboo		Pc27	
128	VIC	SG	Mirboo	C10	Pc59	19
129	VIC	SG	Mirboo	C6	Pc27	
130	VIC	SG	Mirboo	C6		
131	VIC	SG	Mirboo	C6	Pc59	
132	VIC	SG	Mirboo	C1		
133	VIC	SG	Mirboo	C10		
134	VIC	SG	Mirboo	C6		
135	VIC	SG	Mirboo	C1	Pc27	
136	VIC	SG	Mirboo	C6	Pc27	
137	VIC	SG	Mirboo South	C10		
138	VIC	SG	Moe	C10		
139	VIC	SG	Moe	C6	Pc17	
140	VIC	SG	Mullungdung	C1	Pc27	
141	VIC	SG	Narracan	C10	Pc17	
142	VIC	SG	Narracan	C1		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
143	VIC	SG	Narracan	C1		
144	VIC	SG	Narracan	C2		
145	VIC	SG	Narracan	C1	Pc17	
146	VIC	SG	Narracan	C6		
147	VIC	SG	Narracan	C6		
148	VIC	SG	Narracan	C1		
149	VIC	SG	Narracan	C2	Pc27	
150	VIC	SG	Narracan	C2	Pc27	
151	VIC	SG	Narracan	C6	Pc27	
152	VIC	SG	Narracan South	C10	Pc27	
153	VIC	SG	Narracan South	C1	Pc27	
154	VIC	SG	Narracan South	C1		
155	VIC	SG	Narracan South	C1		
156	VIC	SG	Narracan South	C10		
157	VIC	SG	Narracan South	C1	Pc27	
158	VIC	SG	Nerrena	C1	Pc27	
159	VIC	SG	Nerrena	C1	Pc27	16
160	VIC	SG	Nerrena	C6	Pc27	
161	VIC	SG	Nerrena	C10	Pc27	
162	VIC	SG	Nerrena	C1	Pc4	
163	VIC	SG	Nerrena		Pc4	
164	VIC	SG	Rosedale	C6	Pc27	16
165	VIC	SG	Rosedale	C6		
166	VIC	SG	Tarwin	C10		
167	VIC	SG	Tarwin	C10	Pc4	
168	VIC	SG	Tong Bong	C2	Pc57	17
169	VIC	SG	Tong Bong	C6		
170	VIC	SG	Tong Bong	C1		
171	VIC	SG	Tong Bong	C1	Pc17	18
172	VIC	SG	Tong Bong	C6		
173	VIC	SG	Tong Bong	C1		
174	VIC	SG	Tong Bong	C1		
175	VIC	SG	Tong Bong	C6		
176	VIC	SG	Traralgon	C1	Pc27	16
177	VIC	SG	Traralgon	C1		
178	VIC	SG	Traralgon	C2		
179	VIC	SG	Waratah	C7	Pc27	16
180	VIC	SG	Waratah	C10	Pc27	
181	VIC	SG	Waratah	C1	Pc27	
182	VIC	SG	Waratah North	C6	Pc26	
183	VIC	SG	Waratah North	C6		
184	VIC	SG	Willung		Pc17	
185	VIC	SG	Willung	C2		
186	VIC	SG	Willung	C10		
187	VIC	SG	Willung	C2		
188	VIC	SG	Willung	C10		
189	VIC	SG	Willung	C6		
190	VIC	SG	Wonga Wonga	C1		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
191	VIC	SG	Wonga Wonga	C10		
192	VIC	SG	Wonga Wonga	C1		
193	VIC	SG	Wonga Wonga	C2		
194	VIC	SG	Wonga Wonga	C1		
195	VIC	SG	Wonwron	Pc17		18
196	VIC	SG	Wonwron	C10		
197	VIC	SG	Wonwron	C6		
198	VIC	SG	Wonwron	C10		
199	VIC	SG	Wonwron	C2		
200	VIC	SG	Wonwron	C1	Pc27	
201	VIC	SG	Wonwron	C1		
202	VIC	SG	Wonwron	C2	Pc27	
203	VIC	SG	Wonwron	C2		
204	VIC	SG	Wonwron	C2		
205	VIC	SG	Wonwron	C10	Pc27	
206	VIC	SG	Wonyip	C10	Pc27	
207	VIC	SG	Woodside	Pc17		
208	VIC	SG	Woodside	C6	Pc17	
209	VIC	SG	Woorarra	C2		
210	VIC	SG	Yanakie	C6	Pc27	
211	VIC	SG	Yanakie South	C10	Pc27	
212	VIC	SG	Yanakie South	C1	Pc27	
213	VIC	SG	Yanakie South	C2	Pc27	
214	VIC	SG	Yanakie South	Pc27		
215	VIC	SG	Yanakie South	Pc27		
216	VIC	SG	Yarram Yarram	C6	Pc27	
217	VIC	SG	Yarram Yarram	C6	Pc27	
218	VIC	SG	Yinnar	C10		
219	VIC	SG	Yinnar	C6	Pc27	16
220	VIC	SG	Yinnar	C2		
221	VIC	SG	Yinnar	C1		
222	VIC	SG	Yinnar	C6	Pc27	
223	VIC	SG	Yinnar	C1		
224	VIC	SG	Yinnar	C1	Pc58	
225	VIC	SG	Yinnar	C10	Pc27	
226	VIC	SG	Yinnar	C1		
227	VIC	SG	Yinnar	C1	Pc27	
228	VIC	SG	Yinnar	C1		
229	VIC	SG	Yinnar	C1		
230	VIC	SG	Yinnar	C10	Pc17	
231	VIC	SG	Yinnar	C1		
232	VIC	SG	Yinnar	C6	Pc17	
233	VIC	SG	Yinnar	C1		
234	VIC	SG	Yinnar	C1		
235	VIC	SG	Yinnar	C1	Pc27	
236	VIC	SG	Yinnar	C10	Pc27	
237	VIC	RI	Boole Poole	C7		
238	VIC	RI	Boole Poole	C7		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
239	VIC	RI	Boole Poole	C1		
240	VIC	RI	Boole Poole	C7	Pc27	
241	VIC	RI	Boole Poole	C7	Pc27	
242	VIC	RI	Boole Poole	C7	Pc27	
243	VIC	RI	Boole Poole	C7	Pc27	
244	VIC	RI	Boole Poole	C7	Pc27	16
245	VIC	RI	Boole Poole	C7	Pc27	
246	VIC	RI	Boole Poole	C7		
247	VIC	RI	Boole Poole	Pc27		
248	VIC	RI	Boole Poole	C7		
249	VIC	RI	Boole Poole	C7		
250	VIC	RI	Boole Poole	C7	Pc27	
251	VIC	RI	Boole Poole	C7		
252	VIC	RI	Boole Poole	Pc27		
253	VIC	RI	Boole Poole	C7	Pc27	
254	VIC	RI	Boole Poole	C7		
255	VIC	RI	Boole Poole	C7	Pc27	16
256	VIC	RI	Boole Poole	C7	Pc27	16
257	VIC	RI	Boole Poole	C7	Pc27	
258	VIC	RI	Boole Poole	C7		
259	VIC	RI	Boole Poole	C7	Pc27	16
260	VIC	RI	Boole Poole	C7	Pc27	
261	VIC	RI	Boole Poole	C7	Pc27	
262	VIC	RI	Boole Poole	C7	Pc27	
263	VIC	RI	Boole Poole	C7		
264	VIC	RI	Boole Poole	C7	Pc27	16
265	VIC	RI	Boole Poole	C7		
266	VIC	RI	Boole Poole	C7		
267	VIC	RI	Boole Poole	C7	Pc27	16
268	VIC	RI	Boole Poole	C7	Pc27	
269	VIC	RI	Boole Poole	C7		
270	VIC	FI	French Island	C13		
271	VIC	FI	French Island	C13		
272	VIC	FI	French Island	C13		
273	VIC	FI	French Island	C13		
274	VIC	FI	French Island	C13		
275	VIC	FI	French Island	C13		
276	VIC	FI	French Island	C13		
277	VIC	FI	French Island	C13		
278	VIC	GIPPS	Mallacoota	C13		
279	VIC	GIPPS	Mallacoota	C13	Pc27	
280	VIC	GIPPS	Wau Wauka West	C13		
281	VIC	GIPPS	Wuk Wuk	C13	Pc27	
282	VIC	OTW	Otway	C13		
283	VIC	OTW	Otway	C13		
284	VIC	OTW	Otway	C13		
285	VIC	OTW	Otway	C13		
286	VIC	OTW	Otway	C13		

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
287	VIC	OTW	Otway	C13		
288	VIC	OTW	Otway	C13		
289	VIC	OTW	Otway	C13	Pc27	
290	VIC	OTW	Otway	C13	Pc27	
291	VIC	OTW	Otway	C13		
292	VIC	OTW	Otway	C13		
293	VIC	OTW	Otway	C13	Pc27	
294	VIC	OTW	Otway	C13		
295	VIC	OTW	Otway	C13		
296	VIC	OTW	Otway	C13		
297	VIC	OTW	Otway	C13	Pc27	16
298	VIC	OTW	Otway	C13		
299	VIC	OTW	Otway	C13		
300	VIC	OTW	Otway	C13		
301	VIC	OTW	Otway	C13		
302	VIC	OTW	Otway	C13		
303	VIC	OTW	Otway	C13		
304	VIC	OTW	Otway	C13		
305	VIC	OTW	Otway	C13		
306	VIC	OTW	Otway	C13		
307	VIC	OTW	Otway	C13	Pc27	16
308	VIC	OTW	Otway	C13		
309	VIC	OTW	Otway	C13		
310	VIC	OTW	Otway	C13		
311	VIC	OTW	Otway	C13	Pc27	16
312	VIC	OTW	Otway	C13		
313	VIC	OTW	Otway	C13		
314	VIC	OTW	Otway	C13		
315	VIC	OTW	Otway	C13		
316	VIC	OTW	Otway	C13		
317	VIC	OTW	Otway	C13		
318	VIC	OTW	Otway	C13		
319	VIC	OTW	Otway	C13		
320	VIC	OTW	Otway	C13		
321	VIC	OTW	Otway	C13		
322	VIC	OTW	Otway	C13		
323	VIC	OTW	Otway	C13		
324	VIC	OTW	Otway	C13		
325	VIC	OTW	Otway	C13	Pc27	
326	VIC	OTW	Otway	C13		
327	VIC	OTW	Otway	C13		
328	VIC	OTW	Otway	C13		
329	VIC	OTW	Otway	C13		
330	VIC	OTW	Otway	C13		
331	VIC	OTW	Otway	C13		
332	NSW	SENSW	Abercrombie	C9	Pc19	15
333	NSW	SENSW	Lucas	C9	Pc55	13
334	NSW	SENSW	Murrah	C3	Pc17	14

ID	State	Region	Parish	BAPS cluster	mtDNA control region haplotype	mtDNA concatenated haplotype
335	NSW	SENSW	Murrah	C3	Pc17	14
336	NSW	SENSW	Murrah	C3		
337	NSW	SENSW	Ooranook	C3	Pc17	14
338	NSW	SENSW	Tanja	C3	Pc17	14
339	NSW	SENSW	Tanja	C3	Pc17	14
340	NSW	SENSW	Tanja	C3	Pc17	14
341	NSW	SENSW	Tanja		Pc17	14
342	NSW	SENSW	Wangrah	C9	Pc19	15
343	NSW	SENSW	Wangrah	C9	Pc19	15
344	NSW	SENSW	Wangrah		Pc19	15
345	NSW	SENSW	Wangrah	C9	Pc19	15
346	NSW	NENSW	Albert	C12		
347	NSW	NENSW	Booral	C12		
348	NSW	NENSW	Burrawan	C12		
349	NSW	NENSW	Camden Haven	C12		
350	NSW	NENSW	Cowangara	C12		
351	NSW	NENSW	Macquarie	C12		
352	NSW	NENSW	Macquarie	C12		
353	NSW	NENSW	Macquarie	C12	Pc4	
354	NSW	NENSW	Macquarie	C12	Pc27	11
355	NSW	NENSW	Macquarie	C12	Pc27	11
356	NSW	NENSW	Macquarie	C12	Pc3	12
357	NSW	NENSW	Macquarie	C12	Pc3	12
358	NSW	NENSW	Macquarie	C12	Pc27	11
359	NSW	NENSW	Macquarie	C12	Pc3	12
360	NSW	NENSW	Macquarie	C12		
361	NSW	NENSW	Macquarie	C12		
362	NSW	NENSW	Macquarie	C12		
363	NSW	NENSW	Milbrodale	C11	Pc4	10
364	NSW	NENSW	Milli	C12	Pc54	8
365	NSW	NENSW	Tomaree	C12	Pc4	9
366	QLD	SEQLD	?	C4	Pc31	5
367	QLD	SEQLD	?		Pc46	
368	QLD	SEQLD	?	C14	Pc7/Pc15	
369	QLD	SEQLD	?		Pc7/Pc15	
370	QLD	SEQLD	Boyd	C8	Pc7/Pc15	2
371	QLD	SEQLD	Boyd	C8	Pc7/Pc15	
372	QLD	SEQLD	Boyd	C8	Pc7/Pc15	
373	QLD	SEQLD	Ferguson		Pc14	
374	QLD	SEQLD	Gatton	C5	Pc28	7
375	QLD	SEQLD	Goodna	C14	Pc14	3
376	QLD	SEQLD	Goodna	C14	Pc40	4
377	QLD	SEQLD	Goodna	C5	Pc14	
378	QLD	SEQLD	Lockyer		Pc34	6
379	QLD	SEQLD	Lockyer	C12	Pc34	6
380	QLD	SEQLD	Murphy		Pc37	
381	QLD	SEQLD	Tingalpa	C8	Pc7/Pc15	2
382	QLD	SEQLD	Tingalpa	C14	Pc36	1

Table S2 Fine scale genetic differentiation, using microsatellite data, in South Gippsland using D_{jost} (below the diagonal) and F_{ST} (above the diagonal). 95% confidence intervals for each estimate are shown in parentheses. Significant values are shown in bold.

		GENELAND cluster					
		1	2	3	4	5	6
GENELAND cluster	1	-	0.01 (-0.05 - 0.11)	0.03 (-0.03 - 0.12)	0.05 (0.00 - 0.13)	0.12 (0.05 - 0.21)	0.09 (0.03 - 0.18)
	2	0.00 (0.00 - 0.14)	-	0.00 (-0.05 - 0.07)	0.00 (-0.03 - 0.06)	0.04 (-0.01 - 0.10)	0.04 (0.00 - 0.10)
	3	0.02 (0.01 - 0.17)	0.00 (0.00 - 0.09)	-	0.03 (0.00 - 0.07)	0.06 (0.02 - 0.13)	0.05 (0.02 - 0.11)
	4	0.02 0.01 - 0.14)	0.00 (0.00 - 0.06)	0.01 (0.01 - 0.07)	-	0.03 (0.01 - 0.05)	0.03 (0.01 - 0.06)
	5	0.09 (0.05 - 0.20)	0.01 (0.01 - 0.09)	0.03 (0.02 - 0.10)	0.02 (0.01 - 0.05)	-	0.03 (0.00 - 0.06)
	6	0.05 (0.03 - 0.19)	0.03 (0.02 - 0.12)	0.02 (0.02 - 0.10)	0.02 (0.01 - 0.06)	0.01 (0.01 - 0.05)	-

Table S3 Variable sites for 2955 bp concatenated mtDNA made up of the cytochrome B gene (933 bp), a section of DNA spanning part of the NADH dehydrogenase 5 and 6 genes (1381 bp) and the mtDNA control region (641 bp)

Region	Haplotype	n	mtDNA control region (641 bp)																														cytochrome B (933 bp)								NADH dehydrogenase (1381 bp)																	
			7	22	43	68	113	114	122	190	239	248	267	275	278	335	348	361	363	365	383	393	403	437	483	496	540	634	637	660	760	787	918	948	999	1089	1232	1341	1389	1404	1482	1637	1660	1790	1792	2233	2325	2371	2586	2614	2755	2769	2796	2817	2860			
QLD (SE)	Hap01	1	G	T	C	C	A	C	T	C	T	A	C	T	A	A	T	C	T	T	T	G	T	G	C	A	A	G	T	C	C	A	T	A	A	T	T	C	A	A	T	T	C	G	A	C	T	T	C	C	C	C	T	T	T			
	Hap02	2	T			
	Hap03	1	C	T				
	Hap04	1	G	T				
	Hap05	1	.	.	T	T	T	C	.	.	C	T	G	.	.	C		
	Hap06	2	.	.	T	T	T	C	G	.	C	T	C		
	Hap07	1	.	.	T	T	.	T	T	C	.	G	.	T	C	
NSW (NE)	Hap08	1	.	.	T	T	.	.	C	.	.	.	T	C	.	.	.	T	C	A	T	.	.	.	C	T	T	G	A	G	G	C	G	T		
	Hap09	1	.	.	T	T	G	.	C	.	.	.	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	G	.	.	.	T	T		
	Hap10	1	.	.	T	T	G	.	C	.	.	.	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	.	.	G	G	C	.	.	.	G	.	.	.	T	.	.	.	C	.	.			
	Hap11	3	.	.	T	T	G	.	C	.	.	G	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	.	.	G	G	C	.	.	.	G	.	C	C	.	.	
	Hap12	3	.	.	T	T	G	.	C	.	.	.	T	C	.	.	.	T	C	.	.	A	C	T	T	G	A	G	G	C	.	.	G	G	.	.	.	G	.	C	C	.	.	
NSW (SE)	Hap13	1	.	.	T	T	G	.	C	T	.	.	T	C	.	.	.	T	C	C	G	.	C	T	T	G	A	G	G	C	C	G	T	
	Hap14	7	A	C	T	T	.	.	C	.	.	.	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	T	A	G	T	
	Hap15	5	.	.	T	T	.	.	C	.	.	G	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	T	A	G	
VIC	Hap16	15	.	.	T	T	G	.	C	.	.	G	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	G	C
	Hap17	2	.	.	T	T	G	.	C	.	.	G	T	C	.	.	.	T	C	A	C	T	T	G	A	G	G	C	G	C		
	Hap18	2	.	.	T	T	G	.	C	.	.	G	T	C	.	.	.	T	C	.	C	C	T	T	G	A	G	G	C	G	C			
	Hap19	4	A	C	T	T	.	.	C	.	.	.	T	C	.	.	.	T	C	C	T	T	G	A	G	G	C	T	A	G			
	Hap20	1	A	C	T	T	.	.	C	.	.	.	T	C	.	.	.	T	C	.	.	.	C	.	.	.	C	T	T	G	A	G	G	C	.	T	T	A	G			

Table S4 Pairwise genetic differentiation (D_{jost} below the diagonal and F_{ST} above the diagonal), from microsatellite data, between koala populations across Australia, with 95% confidence intervals shown in parentheses.

	QLD (SE)	NSW (NE)	NSW (SE)	VIC (SG)	VIC (RI)	VIC (FI)
<i>n</i>	12	23	12	224	31	61
QLD (SE)	-	0.11 (0.06 - 0.18)	0.16 (0.12 - 0.22)	0.26 (0.24 - 0.29)	0.31 (0.28 - 0.35)	0.34 (0.32 - 0.38)
NSW (NE)	0.27 (0.21 - 0.48)	-	0.20 (0.15 - 0.25)	0.24 (0.22 - 0.27)	0.28 (0.25 - 0.32)	0.32 (0.29 - 0.35)
NSW (SE)	0.36 (0.30 - 0.52)	0.50 (0.44 - 0.60)	-	0.22 (0.20 - 0.26)	0.27 (0.23 - 0.31)	0.34 (0.31 - 0.38)
VIC (SG)	0.54 (0.48 - 0.63)	0.54 (0.49 - 0.60)	0.36 (0.31 - 0.45)	-	0.08 (0.06 - 0.10)	0.12 (0.11 - 0.14)
VIC (RI)	0.64 (0.60 - 0.70)	0.64 (0.58 - 0.70)	0.34 (0.28 - 0.46)	0.04 (0.03 - 0.07)	-	0.12 (0.09 - 0.16)
VIC (FI)	0.61 (0.55 - 0.67)	0.60 (0.55 - 0.66)	0.49 (0.42 - 0.60)	0.10 (0.08 - 0.12)	0.07 (0.05 - 0.10)	-

Chapter 9 | foreword

Chapter eight highlights the importance of conserving the koala population in South Gippsland. Two pathogens afflicting koala populations are, however, potentially of conservation concern for the South Gippsland koala population; *Chlamydia pecorum* and koala retrovirus (KoRV). To date, data regarding the prevalence of *C. pecorum* and KoRV in the South Gippsland koala population has been extremely limited. Large sample sizes can be difficult to obtain using animal capture, while opportunistic sampling may increase the risk of biased results.

Non-invasive methods described in chapter six were used in chapter nine to investigate the prevalence of *C. pecorum* and KoRV across the region, with comparisons to a range of sampled reference populations. Surveys for the presence of these pathogens are based on large sample sizes ($n=176$ and $n=142$, respectively) of the wild koala population in South Gippsland, demonstrating the power of non-invasively sampled DNA to provide large, informative data sets with relative ease. Pathogen prevalence presented in the following two chapters represents the first widespread survey of these pathogens in South Gippsland, providing important baseline data for future studies.



Chapter 9

Using non-invasive sampling methods to determine the prevalence and distribution of *Chlamydia pecorum* and koala retrovirus in the South Gippsland koala population



Submitted to *Wildlife Research*



Chapter 9

Using non-invasive sampling methods to determine the prevalence and distribution of *Chlamydia pecorum* and koala retrovirus in the South Gippsland koala population

Abstract

Pathogenic infections are an important consideration for the conservation of native species. Obtaining data for pathogenic infections in wild natural populations can, however, be expensive and difficult. The koala (*Phascolarctos cinereus*) is infected by two major pathogens potentially impacting population health: urogenital infection with *Chlamydia pecorum* and koala retrovirus (KoRV), though there is a lack of data available regarding the impacts of these pathogens at the population level. Pathogen data for the wild South Gippsland koala population is essentially absent. Non-invasive methods of data collection have the potential to provide greater opportunities for widespread data collection and population monitoring.

This study aims to provide preliminary prevalence and genetic variant data for *C. pecorum* ($n=176$) and KoRV ($n=142$) in the South Gippsland koala population using non-invasive sampling of koala faeces (scats).

DNA isolated from scats was used to identify individuals and detect the presence of *C. pecorum* and KoRV. Shelter animals from South Gippsland and individuals from reference populations at Cape Otway, Raymond Island, Mallacoota (in Victoria), South East New South Wales, North East New South Wales and South East Queensland were also sampled.

C. pecorum and KoRV were detected in 61% and 27% of South Gippsland individuals tested, respectively. Compared to the wild South Gippsland population, shelter animals from South Gippsland were infected with *C. pecorum* at a similar rate while KoRV-A infection tended to be more common in euthanased shelter animals from South Gippsland. Six genetic variants of *C. pecorum* (B, C, F, I, M and O) and two genetic variants of KoRV-A (KV01 and KV03) were detected in South Gippsland.

Continued monitoring of the prevalence of *C. pecorum* and KoRV-A in the South Gippsland koala population will be important for the conservation of this genetically unique and diverse koala population.

Non-invasive genetic sampling from koala scats is a powerful method for obtaining data regarding pathogen prevalence and genetic diversity in wild populations. The use of non-invasive methods for the study of pathogens may assist in determining population level impacts and filling research gaps that may be difficult to achieve using traditional sampling methods.

Introduction

Infectious diseases in wildlife have the potential to contribute to population decline (McCallum 2012). The consideration of pathogenic organisms in conservation biology and the prediction of extinction risk is therefore important (Gerber *et al.* 2005; McCallum 2012). Impacts of a particular pathogen on individuals are often clear, but determining whether the pathogen has a significant impact at the population level can be more difficult (McCallum *et al.* 2017).

The koala (*Phascolarctos cinereus*) is an arboreal marsupial inhabiting eucalypt forests of Australia's east (Martin & Handasyde 1999). Two pathogens, *Chlamydia pecorum* and koala retrovirus (KoRV), infect koalas and are frequently reported contributors to population decline. However, there is lack of information regarding the impact of these pathogens at the population level (Grogan *et al.* 2017; McCallum *et al.* 2017).

One major reason for the lack of population level studies involving pathogens is the need for specialist expertise, such as handlers and veterinarians, to capture and sample animals. Such expertise is expensive. A 2006 study involving koala capture and anaesthetisation reported the cost of capture and veterinarian teams to average \$1362 per koala caught (Radford *et al.* 2006). Non-invasive sampling, where samples are obtained from discarded sources such as scats, provides an alternative. Both *C. pecorum* and KoRV can be detected in DNA isolated from scats (Wedrowicz *et al.* 2016) providing a means for systematic sampling of large numbers of koalas at a reduced cost.

Chlamydia pecorum

The genus *Chlamydia* comprises gram negative, intracellular bacteria associated with a range of diseases in their hosts, which include humans, ruminants, marsupials and birds (Rank &

Yeruva 2014). Along with koalas, *C. pecorum* also commonly affects populations of cattle, swine and sheep. In koalas, *C. pecorum* infects both ocular and urogenital tissues, with significant pathological outcomes (Blanshard & Bodley 2008). The bacteria can rapidly spread within populations, with potential negative consequences for koala fecundity and health (Santamaria & Schlagloth 2016). In particular, *C. pecorum* infections of the urogenital tract (UGT) can lead to sterility (Obendorf & Handasyde 1990), potentially contributing to population decline. Animals not previously exposed to *C. pecorum* may be more susceptible to severe infections (Martin & Handasyde 1990). The faecal-oral route is the main mode of *Chlamydia* transmission in most animal hosts (Rank & Yeruva 2014), but sexual transmission is considered to be the primary route of transmission in koalas. It is possible that *C. pecorum* is also transmitted via the faecal-oral route in koalas (Waugh *et al.* 2016), however this is yet to be demonstrated.

C. pecorum variants are classified according to differences in the nucleotide sequence of the *ompA* gene, which encodes the major outer membrane protein. Fourteen genotypes of *C. pecorum* have been reported in koalas to date, designated by the letters A to N (Addendum Table A2) (Jackson *et al.* 1997; Higgins *et al.* 2012; Kollipara *et al.* 2013; Legione *et al.* 2016b). Different strains of *C. pecorum* may have varying levels of pathogenicity and immune responses may be specific to the infecting strain (Mohamad *et al.* 2008; Mohamad *et al.* 2014). The introduction of unfamiliar strains of *C. pecorum* to a population could have negative health impacts (Waugh *et al.* 2016) and may be of concern for koala populations, since koalas are commonly moved from their area of origin for management purposes (Menkhorst 2008; Santamaria & Schlagloth 2016) and by wildlife carers after rehabilitation in shelters (Guy & Banks 2012).

Kollipara *et al.* (2013) found *C. pecorum* prevalence to range between 20% and 61% in wild populations in the north of Australia, with genotype F being the most widespread. *Chlamydia*

is known to be present in populations descended from Phillip Island individuals, while French Island koalas (and certain populations solely established by individuals translocated from French Island) are not known to suffer from chlamydial disease, although very low levels of infection have been reported (Emmins 1996; Legione *et al.* 2016a; Legione *et al.* 2016b). Legione *et al.* (2016b) found *C. pecorum* prevalence in southern populations south ranged from 1% to 46%, with genotype B the dominant strain in western Victoria and Raymond Island, and genotypes C and F most common in Gippsland.

Koala retrovirus (KoRV)

KoRV was first detected in koalas at the turn of the last century (Hanger *et al.* 2000), and is known to have been present in northern Australian koala populations since at least the late 1800s (Ávila-Arcos *et al.* 2013). KoRV may be either exogenous or endogenous (Tarlinton *et al.* 2005; Simmons *et al.* 2012). Endogenous infection results from the insertion of KoRV into germ cells and can therefore be transmitted vertically, to offspring, via Mendelian inheritance (Tarlinton *et al.* 2005; Simmons *et al.* 2012). Integrated virus is capable of producing active virus, so endogenous KoRV is also believed to be transmitted horizontally, between individuals (Tarlinton *et al.* 2005). To date, nine genetic variants (A, B/J, C–I) of KoRV have been identified (Chappell *et al.* 2016), with KoRV-A the most widespread.

The effects of KoRV infection on koala health are currently not clear. In other species, gibbon ape leukaemia virus (which shares a close phylogenetic relationship with KoRV) causes leukaemia in gibbons (Hanger *et al.* 2000) while in cats, the feline leukaemia virus can result in the development of tumours, immunodeficiency and haematopoietic disorders, the most common symptom at initial presentation being anaemia (Hartmann 2012). In koalas there is evidence that KoRV can also cause immunosuppression and cancers such as lymphoma and leukaemia (Tarlinton *et al.* 2005). Koalas with clinical chlamydiosis, a disease sometimes

associated with immunosuppression, tend to have higher KoRV loads (Tarlinton *et al.* 2005) and, in koalas co-infected with *C. pecorum* and KoRV, an increased incidence of urogenital tract disease has been documented (Legione *et al.* 2017).

KoRV-A appears to be endogenous in the north of the koala's range with all koalas sampled in northern New South Wales and Queensland testing positive for KoRV-A provirus (Simmons *et al.* 2012). In southern koala populations (Victoria and South Australia) a proportion of the population is uninfected and the prevalence of KoRV-A is highly variable, ranging from 0% to 69% (Simmons *et al.* 2012; Legione *et al.* 2017).

The number of proviral KoRV-A copies detected per cell also varies greatly between populations in the north and south, with an average of 165 copies per cell in Queensland, one copy per cell in some Victorian koalas and fewer than 0.001 copies per cell in other Victorian koalas (Simmons *et al.* 2012). This finding suggests that, while KoRV-A may be endogenous in some Victorian koalas, many KoRV-A infections exist only in the exogenous form (Simmons *et al.* 2012). In Victoria, infection with KoRV-A, has been found to be significantly associated with low body condition scores and the presence of 'wet bottom', resulting from chronic cystitis (Legione *et al.* 2017), indicating that KoRV-A is likely to be having a negative impact on the health of wild Victorian koala populations.

Sampling for pathogen detection

The reported prevalence of pathogenic infections may not reflect the rate of infection in the living population if samples are largely obtained from sick, injured or deceased individuals. While opportunistic sampling from wildlife shelters or road kill is often the most viable option for studying pathogens and obtaining prevalence data, the potential bias associated with this type of sampling and an inability to obtain comparative data from the wild population can limit the conclusions that may be drawn from such studies.

A remnant koala population, not derived from the translocation of island individuals, remains in the South Gippsland region of Victoria (Menkhorst 2004; Lee et al. 2011; Chapter 8, Wedrowicz *et al.* in review 2). This population is genetically differentiated from, and more diverse than, other southern koala populations (Menkhorst 2004; Lee et al. 2011; Chapter 8, Wedrowicz *et al.* in review 2) and is, therefore, of high conservation value. The prevalence of *C. pecorum* and KoRV-A in the region's wild koala population is not known. In this study, we therefore use non-invasive genetic sampling to investigate the prevalence and spatial distribution of *C. pecorum* and KoRV-A in the South Gippsland koala population and selected reference populations.

Methods

Sample Collection

The focal study area was the South Gippsland region in Victoria, Australia, which covers an area of around 6,000 square kilometres (Fig. 1, Fig. 2). Koalas were also sampled at other sites in Victoria including Raymond Island (koalas of Phillip Island origin), Cape Otway (koalas of French Island origin), Mallacoota (koalas of French Island origin) and interstate koala populations from south east New South Wales (SENSW), north east New South Wales (NENSW) and south east Queensland (SEQLD) (Fig. 1). Shelter animals from the Southern Ash Wildlife Shelter (SAWS), Rawson, Victoria were also sampled and included individuals originating from both South Gippsland and Central Gippsland (around the Rawson Township). Scat samples were collected following the protocol described by Wedrowicz *et al.* (2013) where scats are collected and stored on toothpicks inserted into the side of the scat. Spatial data for scat samples collected from wild populations were recorded using a handheld GPS. Ear biopsies ($n=44$, collected by SAWS) from individuals which had been euthanased, usually without being admitted, were also analysed for the presence of KoRV-A.

Different sample sets were used for the *C. pecorum* and KoRV-A studies. The *C. pecorum* study involved 336 unique isolates, including 176 South Gippsland samples. In Victoria, reference samples were collected from Raymond Island ($n=26$), Cape Otway ($n=41$), Mallacoota ($n=5$) and shelter individuals from South Gippsland ($n=63$). Interstate reference samples were obtained from south east New South Wales ($n=12$) and south east Queensland ($n=13$) (Fig. 1a).

The KoRV-A study involved 263 unique isolates, including 142 South Gippsland samples. In Victoria, reference samples were obtained from Raymond Island ($n=19$), Cape Otway ($n=11$), and shelter individuals from Central Gippsland ($n=17$) and South Gippsland ($n=61$) while interstate populations sampled included coastal and inland regions of south east New South Wales ($n=12$) and shelter individuals from north east New South Wales ($n=17$) (Fig. 1b).

DNA isolation and screening

The surface of the scats were washed in PBS buffer and DNA was isolated from the wash using either the Qiagen QIAamp[®] DNA Stool Mini Kit as described in Wedrowicz *et al.* (2013) or the Axygen[®] AxyPrep[™] MAG Soil, Stool, and Water DNA Kit. Isolations using the Axygen[®] DNA Kit were carried out following the manufacturer's instructions except that 400 μ L of supernatant was transferred after the centrifugation step and 400 μ L SBW buffer was added to the supernatant (rather than 300 μ L of each). The amount of binding enhancer added was also increased to 15 μ L. DNA was isolated from tissue samples using the DNeasy[®] Blood & Tissue Kit (Qiagen) following the manufacturer's protocol.

Genotypic data were used to select DNA isolates with high DNA quality and were also used to identify duplicate samples, which were removed. Consensus genotypes were obtained from three or four replicate genotypes as described in Wedrowicz *et al.* (2013) and Chapter 8 (Wedrowicz *et al.* in review 2). Genotypes consisted of twelve microsatellite markers, K2.1,

K10.1, Pcv6.1, Pcv2, Pcv6.3, Pcv24.2, Pcv25.2, Pcv30, Pcv31 (Cristescu *et al.* 2009), Phc2, Phc4 and Phc13 (Houlden *et al.* 1996). Amplification and product separation using capillary electrophoresis was conducted at the Australian Genome Research Facility (AGRF), Melbourne, Australia. To minimise the chance of false negatives due to poor DNA quantity and/or quality in samples, only DNA isolates producing a microsatellite genotype with at least eight positive loci were used for pathogen screening. Maternally inherited mitochondrial DNA (mtDNA) control region haplotype data were also obtained as described in Wedrowicz *et al.* (2013) using primers KmtL1 and KmtH2 (Fowler *et al.* 2000) to amplify approximately 700 base pairs of DNA.

Fine scale population structure

Only genotypes with reliable spatial coordinates were used to infer fine scale population structure within the South Gippsland region (Chapter 8, Wedrowicz *et al.* in review 2). The spatial coordinates and genotypic data were analysed with GENELAND in R (Guillot *et al.* 2008), using 1,500,000 iterations inclusive of a 500,000 iteration burn-in, a thinning parameter of 100 and the correlated allele frequency model. The distribution of infection was subsequently mapped to the population clusters inferred by GENELAND (Chapter 8, Wedrowicz *et al.* in review 2). The natural breaks (Jenks) method in ArcGIS was used to categorise the prevalence of infection for each population cluster into three categories of prevalence greater than 0%.

Detection of Chlamydia pecorum and classification of ompA sequences

DNA isolates were screened for the presence of *C. pecorum* using a real time PCR assay targeting a 76 bp region of the *Chlamydia* outer membrane protein A (*ompA*) gene (Pantchev *et al.* 2010) including TaqMan[®] Exogenous Internal Positive Control (IPC) Reagents. Amplification was carried out using a presence–absence protocol on the Applied Biosystems

Step One Plus instrument (Wedrowicz *et al.* 2016). For a randomly selected subset of positive isolates ($n=61$), approximately 1140 bp of the *C. pecorum ompA* gene was amplified and sequenced as described in Wedrowicz *et al.* (2016).

Sequences with greater than 1% nucleotide difference from previously described genotypes were considered a new genotype and designated a new letter as per Kollipara *et al.* (2013). If the *ompA* sequence had nucleotide differences of less than 1%, sequences were classified as the same genotype (using the same letter) followed by a prime symbol. When several genotypes were detected with less than 1% differences in nucleotide sequence, the genotype letter was designated with both a number and a prime symbol (e.g. B, B'1, B'2 etc.) and referred to as a genotype variant.

KoRV-A PCR and sequencing

Infection with KoRV-A was determined using standard PCR as described in Wedrowicz *et al.* (2016). Standard PCRs utilised KoRV-A specific primers published in Xu *et al.* (2013) alongside koala β -actin primers (Markey *et al.* 2007) to confirm the presence of koala DNA. To investigate potential genetic variants of KoRV-A across broad sample areas, a 1115 bp region of the KoRV-A *env* gene was amplified for a subset of KoRV-A positive samples ($n=17$) using primers KoRV-*env*1-F (5'-AGACGGGAAGTGTCGTTTGG-3') and KoRV-*env*1-R (5'-GGGGGTGAGGCCAGAATTAC-3') (see Wedrowicz *et al.* (2016) for PCR details). Sequences were subsequently aligned and compared using MEGA 6 (Tamura *et al.* 2013).

Results

Chlamydia pecorum

Prevalence of C. pecorum

C. pecorum was detected in nearly half (49%) of all DNA samples tested (166/336). In South Gippsland, *C. pecorum* was detected in 61% (107/176) of individuals sampled. The prevalence of infection in Raymond Island individuals, founded by Phillip Island stock, was high, 81% (21/26), while the prevalence of *C. pecorum* was much lower in populations founded by French island translocations (4.9% (2/41) at Cape Otway and was not detected in any of the five individuals sampled from Mallacoota). Prevalence of *C. pecorum* was 38% (5/13) and 27% (3/11) in NENSW and SEQLD respectively, which was lower than that detected in South Gippsland, although sample sizes were small.

Within South Gippsland there are two regions where population density is higher than in surrounding areas, with a population density around one koala per four hectares, (Allen 2015; R. Appleton, HVP, pers. comm.). The areas of higher population density correspond to population clusters 4 and 5 in Fig. 2. We grouped populations according to low or high koala density and compared prevalence. The prevalence of *C. pecorum* infection was significantly greater ($p=0.0004$) in areas of high koala density (Fig. 2a, regions 4 and 5 compared to surrounding areas, where koala densities are lower (Fig. 2a, all other regions), with infection rates of 77% (51/66) and 49% (52/106), respectively.

ompA diversity

ompA gene sequence data were obtained for 61 samples confirmed positive for *C. pecorum* using real time PCR. In South Gippsland, six *ompA* genotypes were detected: F (42%), B (23%), M (21%), C (9%), I (2%) and one novel genotype designated genotype O (Table 1).

Previously unreported genotype variants were also found in South Gippsland. These included two variants of genotype C (C'1 and C'2) that differed from genotype C reported by Legione *et al.* (2016b) by one and two bases respectively (amino acid sequences of all three C genotypes were identical). Three group B genotype variants (B'4–B'6) and five F genotype variants (F'3–F'7) were also detected. All B and F genotype variants had differing protein sequences, with three to six amino acid differences between the B variants and two to four amino acid differences between the F variants. Another *ompA* sequence with seven base pair differences to genotype I (Kollipara *et al.* 2013) was detected and specified as genotype variant I' (seven amino acid differences were also present). *C. pecorum ompA* sequence data generated in this study are available under GenBank accession numbers KY913821 – KY913837.

The range of *ompA* genotypes and genotype variants found in South Gippsland populations contrasted with the limited range detected in other Victorian populations; all sequenced Raymond Island samples had *ompA* genotype B'1 or B'4 (only one base pair different to *ompA* genotype B), while genotype L was detected in both positive Cape Otway samples.

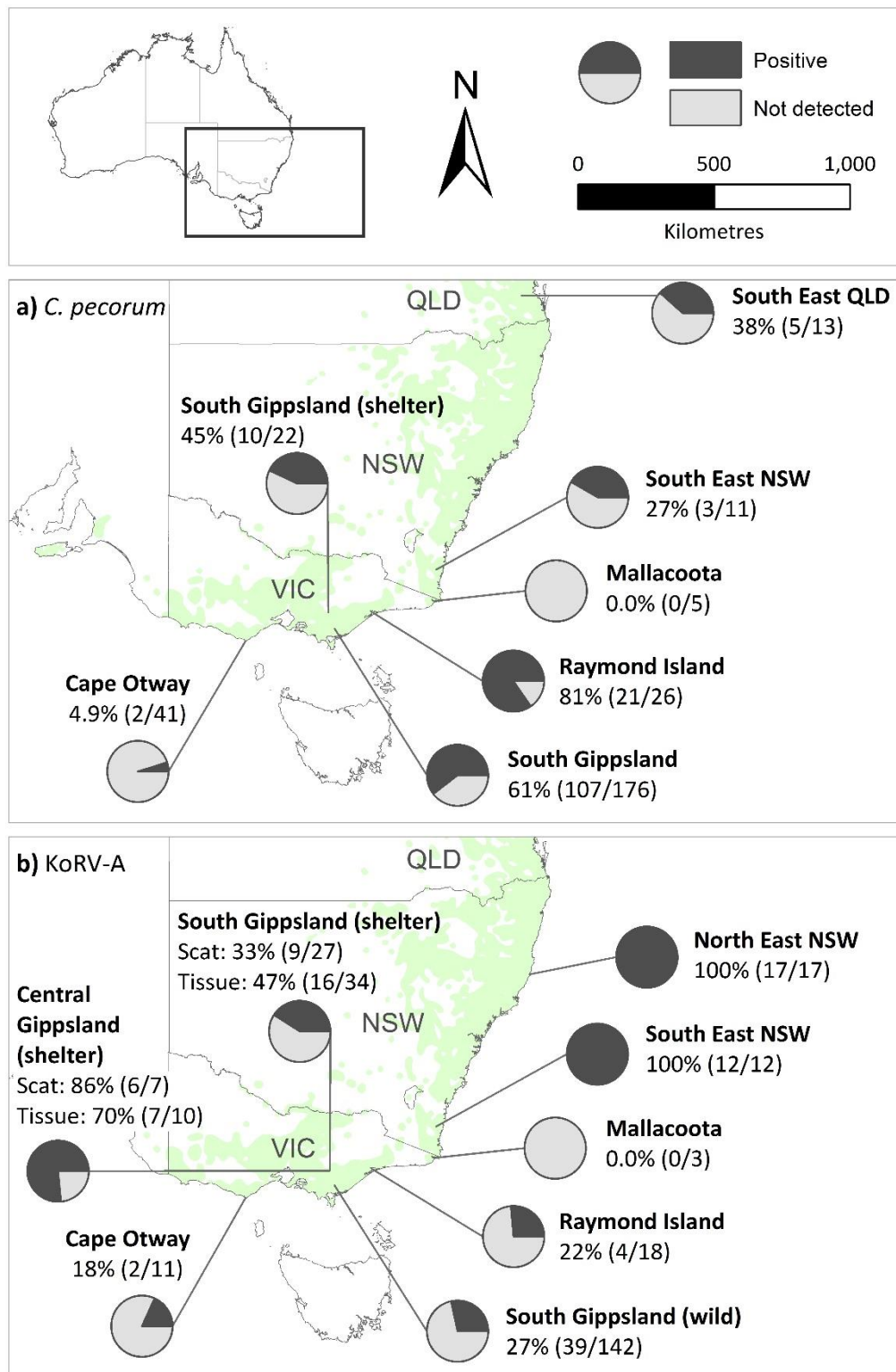


Figure 1 Prevalence of infection with a) *C. pecorum* and b) KoRV detected in DNA isolated from koala scats. The distribution of the koala is shaded green (adapted from Department of the Environment 2015). Dark grey on the pie charts indicates the proportion of individuals sampled for which *C. pecorum* or KoRV was detected while light grey denotes the proportion of individuals sampled in which *C. pecorum* or KoRV was not detected.

Table 1 Summary of the *C. pecorum ompA* genotypes detected in this study

<i>ompA</i> genotype group	<i>ompA</i> genotype variant	SG	RI	OTW	SENSW	SEQLD
A	A'1	-	-	-	-	1
	B'1	-	10	-	-	-
B	B'4	-	2	-	-	-
	B'5	9	-	-	-	-
	B'6	1	-	-	-	-
C	C	-	-	-	-	-
	C'1	3	-	-	-	-
	C'2	1	-	-	-	-
F	F	-	-	-	1	2
	F'3	11	-	-	-	-
	F'4	4	-	-	-	-
	F'5	1	-	-	-	-
	F'6	1	-	-	-	-
	F'7	1	-	-	-	-
I	I'	1	-	-	-	-
L	L	-	-	2	-	-
M	M	9	-	-	-	-
O	O	1	-	-	-	-
Total		43	12	2	1	3

SEQLD: South East Queensland, **SENSW:** South East New South Wales, **OTW:** Cape Otway, **RI:** Raymond Island, **SG:** South Gippsland, **SHC:** Shelter or captive individuals

KoRV

KoRV-A prevalence in southern Australia

The proportion of individuals testing positive for KoRV-A in sampled populations was variable (Fig. 1b). The incidence of KoRV-A infection in Victorian populations sampled ranged from 18% (2/11) at Cape Otway to 22% (4/18) at Raymond Island and 27% (39/142) in the wild South Gippsland population. Differences between the wild South Gippsland, Cape Otway and Raymond Island populations were not significant. All individuals tested in south east NSW were found to be KoRV-A positive (Fig. 1b). The south east NSW population sampled here currently represents the most southern population found to be infected at a rate much higher (100%) than populations from Victoria and South Australia ($p < 0.0005$).

Animals entering shelters tended to be more likely to test positive for KoRV-A than wild animals from the same region (Fig. 1b). In South Gippsland, KoRV-A was detected in 41% (25/61) of shelter animals, compared to 27% (39/142) of individuals sampled in the wild ($p = 0.08$). The prevalence was even greater for shelter koalas originating from Central Gippsland, where 76% (13/17) of individuals were KoRV-A positive (Fig. 1b). The difference in prevalence between shelter animals from South Gippsland (41%) and shelter animals from Central Gippsland (76%) was significant ($p = 0.02$). Within Victorian shelter animals, KoRV-A was detected at similar rates in both scats (86% CG; 33% SG) and tissues (70% CG; 47% SG), indicating comparable detection rates between sample types.

The distribution of KoRV-A infection in South Gippsland

The fine scale distribution of KoRV-A infection in wild South Gippsland koala population clusters is shown in Fig. 2b. Unlike results for *C. pecorum* in the South Gippsland koala population, KoRV-A prevalence in the wild koala population was not related to population

density. KoRV-A prevalence was similar in areas of relatively high koala density (Fig. 2b, population clusters 4 and 5; 31% positive, $n=55$) and areas of lower koala density (Fig. 2b, all population clusters except 4 and 5; 26% positive, $n=84$).

KoRV-A env sequence differences between populations

DNA sequencing identified three unique KoRV-A *env* genotypes: KV01, previously identified by Hanger *et al.* (2000) (Genbank AF151794.2), was detected in samples from northern NSW and in Victorian samples originating from both Central Gippsland ($n=1$) and South Gippsland ($n=2$); KV02, *env* sequence one base pair different to KV01, was found exclusively in south east NSW samples ($n=4$); KV03, *env* sequence seven base pairs different to KV01, was found only in Victorian koalas ($n=8$; Table 2). Sequences obtained from the *env* gene are available under GenBank accession numbers KY979231–KY979233. All base changes were synonymous, except for nucleotide 974 in *env* genotype KV03 (Table 2). The nucleotide change at site 974 from A to C resulted in an amino acid substitution from asparagine in K01 to histidine in K03. The amino acid sequence at sites 324–326 was asparagine-alanine-serine in K01, a motif that is associated with N-linked glycosylation of the asparagine residue (Gavel & Heijne 1990). The amino acid substitution at this site in K03 (resulting in histidine-alanine-serine), may therefore, have potentially resulted in the loss of a glycosylated site.

Interestingly, mitochondrial control region haplotype data showed that individuals with KoRV-A *env* genotype KV03 had the Pc27 haplotype ($n=6$), while individuals with the KoRV-A *env* genotype KV01 had the Pc17 haplotype ($n=2$). There was no association between mitochondrial control region haplotype and KoRV-A prevalence; 27% of individuals with haplotype Pc17 ($n=15$) and 31% of individuals with haplotype Pc27 ($n=90$) were KoRV-A positive.

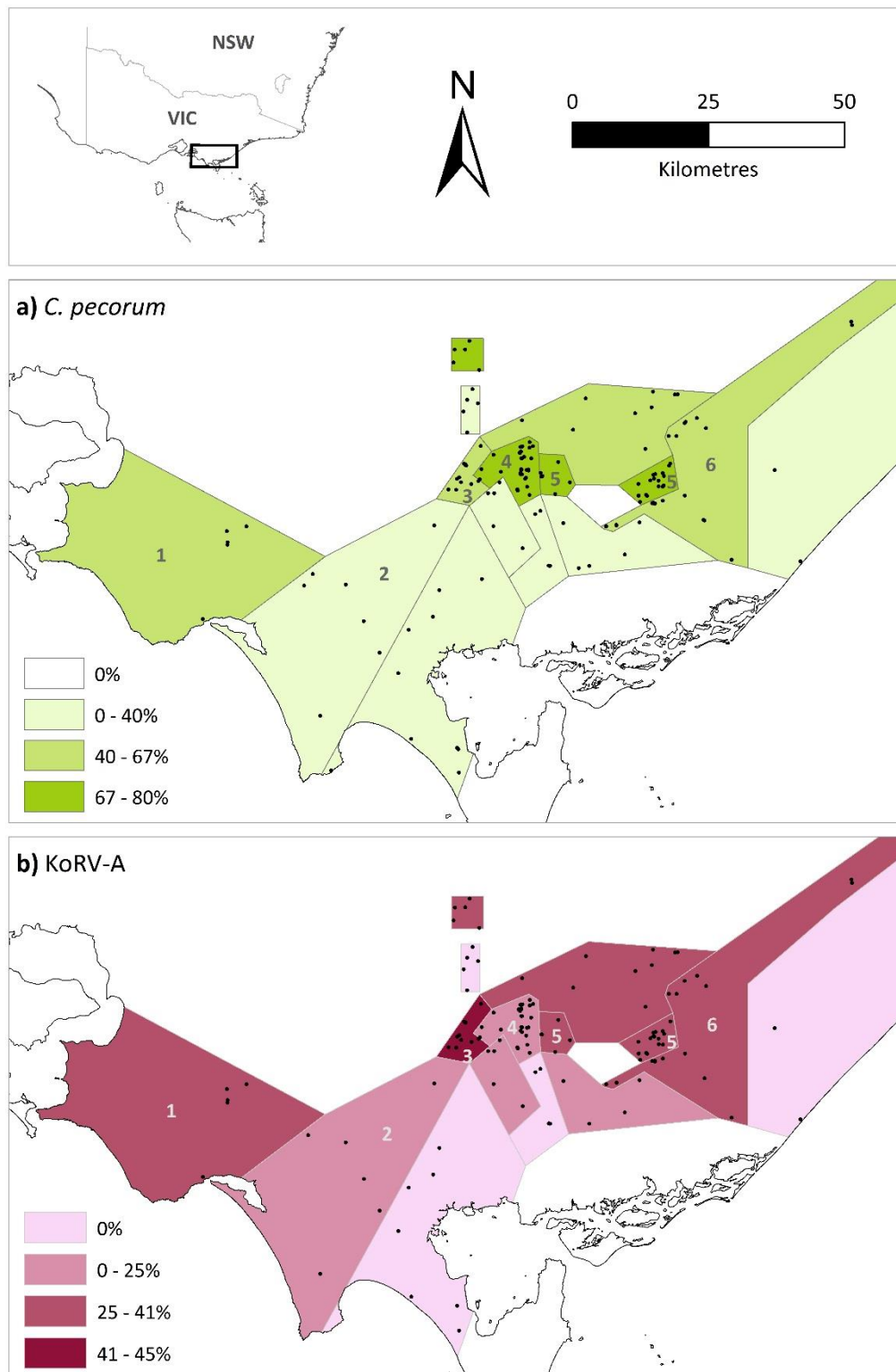


Figure 2 Prevalence of a) *C. pecorum* and b) KoRV infection in South Gippsland according to koala population cluster identified by GENELAND. Clusters with more than 6 individuals are numbered from 1 to 6. Population clusters 4 and 5 are areas of relatively high koala density, while surrounding areas generally have lower densities of koalas. Points on the map represent the location of sampled koalas. White areas of the map represent regions not sampled.

Table 1 Variable sites in KoRV-A *env* sequences and the frequency with which each was detected by population.

	Nucleotide position								Frequency by sample group				
	46	52	82	349	403	499	613	974	SG	CG	RI/OTW	SENSW	NENSW
KV01	C	C	A	A	C	G	G	A	2/6	1/2	-	-	2/2
KV02	A	.	-	-	-	4/4	-
KV03	T	T	G	G	T	A	.	C	4/6	1/2	3/3	-	-

SG: South Gippsland, **CG:** Central Gippsland, **RI/OTW:** Raymond Island, **SENSW:** South East New South Wales, **NENSW:** North East New South Wales.

Prevalence of individuals with both C. pecorum and KoRV

Of the 158 individuals sampled in South Gippsland that were tested for both *C. pecorum* and KoRV-A, neither infection was detected for 52 (33%) individuals, only *C. pecorum* for 62 (39%) individuals, only KoRV-A for 14 (9%) individuals and both *C. pecorum* and KoRV-A for 30 (19%) individuals. Rates of infection between these categories were similar between females and males. However, males were more likely to be free of either infection than females (40% and 22% respectively, $p=0.02$)

Discussion

This study represents the first large-scale investigation of the prevalence and spatial distribution of *C. pecorum* and KoRV-A in the wild South Gippsland koala population, made possible by use of non-invasive DNA sampling from koala scats. The prevalence of infection, and not the prevalence of disease, was estimated in this study. Whether individuals sampled were symptomatic or asymptomatic was not known or not recorded.

Pathogen prevalence

Levels of infection with *C. pecorum* are high within South Gippsland (overall 61%), ranging from 49% in low density areas to 77% in high density areas. Apart from one novel genotype (O), all other genotypes detected in this study are identical or near identical to genotypes previously reported from UGT samples (Kollipara *et al.* 2013; Legione *et al.* 2016b) showing that the same strains of koala *C. pecorum* detected in UGT samples are detected in DNA isolated from scats. Severe chlamydial disease is thought to be more common in koalas from northern Australia compared to those in southern Australia (EaCRC 2011). However, excluding populations derived from French Island individuals (i.e. Cape Otway and Mallacoota in Victoria) where the prevalence of *C. pecorum* is very low (Emmins 1996; Legione *et al.* 2016a), this study showed that the prevalence of *C. pecorum* infection in southern koala populations (South Gippsland and Raymond Island in Victoria) is certainly not less than northern koala populations in New South Wales and Queensland.

Koala populations in which all animals tested positive for KoRV-A were previously identified in northern NSW and all regions further north, in Queensland (Simmons *et al.* 2012). All koalas tested from populations in south east NSW in this study were also found to be KoRV-A positive. The prevalence of KoRV-A in South Gippsland was estimated at 27%, which is higher than the 18% prevalence in wild-ranging Gippsland koalas recently reported by

Legione *et al.* (2017). However, Legione *et al.* (2017) collected samples more broadly across Gippsland, rather than in South Gippsland alone. Rates of infection for free ranging populations at Cape Otway and Raymond Island recorded in this study are in close agreement with those reported by Legione *et al.* (2017) and the prevalence of KoRV-A determined in this study for the Raymond Island population, using scats (4/18) is also comparable to the prevalence reported by Simmons *et al.* (2012), using blood samples from wild individuals.

KoRV-A prevalence in South Gippsland has previously been reported to be as high as 69% (Simmons *et al.* 2012), which is significantly higher than the overall prevalence determined for South Gippsland in this study (27%, $p < 0.0005$). However, Simmons *et al.* (2012) used samples collected opportunistically, primarily from dead South Gippsland animals, so the potential for KoRV-A positive individuals to be overrepresented in shelter or road killed samples may explain this difference.

Comparison of wild and shelter koalas

All shelter individuals tested for *C. pecorum* originated from areas outside of the two higher density populations (i.e. low density areas). *C. pecorum* was detected in 45% of the wildlife shelter koala population while prevalence for *C. pecorum* in the low density areas of South Gippsland was 49%, indicating a similar rate of infection in both shelter and wild populations.

KoRV-A was detected in 41% of shelter individuals originating from South Gippsland and 27% of wild individuals within South Gippsland. Differences in KoRV-A prevalence between shelter and wild animals (as well as between this study and the Simmons *et al.* (2012) study) may reflect negative impacts of KoRV-A on koala health and, subsequently, an over-representation of KoRV-A positive individuals in Victorian koalas affected by road trauma, requiring veterinary treatment, or admitted to shelters. This is supported by Legione *et al.* (2017) who found that koalas in poor health (with low body condition scores) were seven

times more likely to be KoRV-A positive. Further sampling of shelter koalas, along with admission data, such as blood biochemistry, diagnosis and body condition score, would be useful to gain further insight into the effects of KoRV-A on koalas.

Similar prevalence of *C. pecorum* between shelter and wild koalas potentially suggests that *C. pecorum* is not a major cause of mortality in the South Gippsland koala population while higher prevalence of KoRV-A in shelter animals compared to wild koalas suggests that KoRV-A is a potential contributor to mortality in the South Gippsland koala population. Further targeted research is needed to investigate the effects of these pathogens at the population level.

Pathogen genotypes

Different genotypes of *C. pecorum* were noted in both geographically close (e.g. sub groups in South Gippsland) and distant (e.g. Cape Otway, Raymond Island and South Gippsland) koala populations. Virulence of *C. pecorum* strains infecting other animal hosts may vary (Mohamad *et al.* 2014) and the severity of disease may differ depending on the health of the individual animal or the presence of environmental stressors that may impact health and therefore disease susceptibility (Timms 2005; Lunney *et al.* 2012; McAlpine *et al.* 2017). Although a greater amount of research into the pathogenicity of koala *C. pecorum* strains is required, the potential for exposure to new chlamydial strains that may have negative health impacts are important considerations for proposed translocations. For this same reason, it is also important to return rehabilitated individuals to their exact location of origin. Given that asymptomatic infection may permit transmission to others, who may then disseminate foreign strains among the population at the site of release, the quarantine animals from differing locations at wildlife hospitals is also important.

The three KoRV *env* genotypes identified in this study corresponded broadly with sampling location, with KV01 being mostly from northern NSW (but also found in Victoria), KV02 from southern NSW and KV03 from Victoria.

Four potential glycosylation sites were present in the envelope protein sequence identified in KoRV-A genotypes KV01 and KV02 identified in this study (at sites 248–250, N-A-T; 319–321, N-L-T; 325–327, N-A-S and 337–339, N-H-S). The KoRV-A genotype commonly found in Victoria (KV03) had only three of the above named glycosylation motifs due to DNA mutation resulting in the conversion of the asparagine (N) residue at amino acid site 325 to histidine (H) and thus, potentially, loss of a glycosylated site. Given the role of glycosylation in the pathogenesis of many viruses (Vigerust & Shepherd 2007), this may have implications for viral infectivity in Victoria, and could potentially help to explain the differing prevalence rates observed in northern and southern koala populations and the low frequency of KoRV-A endogenisation in southern koala populations.

Pathogen prevalence and population density

The effect of pathogenic infections on host populations is often related to population density (May & Anderson 1979). Population density appears to play a role in the prevalence of infection in the South Gippsland region, with koalas in high-density areas three times more likely to carry *C. pecorum* than those in lower density areas (Fig. 2a). However, fine scale genetic structure (which corresponds to areas of higher density) may also play a part. The presence of *C. pecorum* associated with high population density may also explain the high prevalence of individuals carrying the bacteria on Raymond Island. In contrast, while the density of koalas at Cape Otway is also very high, estimated at around 18 individuals per hectare (Whisson *et al.* 2016), *C. pecorum* was detected in only two individuals from the 41 sampled. This may reflect a very recent introduction of the bacteria to the Cape Otway

population and/or a difference in the strain's (genotype L) pathogenicity. Tolerance of the Cape Otway population to *C. pecorum* is unlikely as rapid dissemination of *Chlamydia* has been recorded in French Island koalas, from which the Cape Otway population was established (Santamaria & Schlagloth 2016). In the Santamaria and Schlagloth (2016) study, French Island individuals were translocated to an area where the resident population was *Chlamydia* positive; on release all koalas were *Chlamydia* free, but almost all (16/17) *Chlamydia* negative animals tested positive for chlamydial antibodies after 19 months, but the *ompA* genotype/s present were not identified.

In contrast to *C. pecorum*, population density did not appear to influence the prevalence of KoRV-A in South Gippsland. Prevalence was similar in areas of relatively high koala density (Fig. 2b, population clusters 4 and 5; 31% positive, $n=55$) and areas of lower koala density (Fig. 2b, all population clusters except 4 and 5; 26% positive, $n=84$). This may suggest that exogenous KoRV-A found in Victoria is not easily transmitted between individuals. This is supported by the finding that KoRV-A infection rates in Victorian koalas have remained stable over a period of about three years, with KoRV-A prevalence on French and Raymond Islands consistently reported at 20 – 30% (Simmons *et al.* 2012; Legione *et al.* 2017).

Future directions

McCallum *et al.* (2017) point out an extreme lack of long term population monitoring and that the relationship between stress and clinical disease may only be clarified by monitoring koalas through time for levels of stress, infection and clinical disease. The ability to obtain a range of information from scat samples including a unique identifying genotype for individual koalas (Chapter 8, Wedrowicz *et al.* in review 2) and infection status for *C. pecorum* and KoRV (Wedrowicz *et al.* 2016) provides a means by which populations may be monitored long term. Additionally, coupling the methods described above with faecal cortisol estimations of stress

(Narayan *et al.* 2013) and observational data such as fertility rates or the presence of “wet bottom” may allow such studies to be conducted without having to interfere with the study animals in any way. Non-invasively studying populations not only minimises animal stress and potential risks to the animal but also reduces costs, potentially allowing the acquisition of larger datasets.

Conclusions

This study highlights the usefulness of non-invasive genetic sampling of koala scats, permitting a comprehensive survey of *C. pecorum* and KoRV-A prevalence in the South Gippsland koala population. Further investigations may provide an indication of the level of impact that these pathogens are having on populations and facilitate the timely implementation of appropriate strategies to limit potential impacts. Given the high prevalence of *C. pecorum* throughout the South Gippsland region and the likelihood of increasing environmental pressures in the future (e.g. climatic changes or habitat shifts; Adams-Hosking *et al.* 2011; González-Orozco *et al.* 2016), the incidence and/or severity of overt disease may increase in the region over the coming years. The evidence that KoRV-A infection is having a negative impact on koalas is also of concern for the conservation of the South Gippsland koala population. Due to the importance of the South Gippsland koala population (Chapter 8, Wedrowicz *et al.* in review 2), it will be vital to monitor these infections in the region over time.

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Chapter 9 | Addendum

The data in chapter 9 gave rise to numerous interesting questions that are in need of further work to draw more solid conclusions. Presentation and discussion of these data were therefore not included in the main chapter but are outlined in the following pages.

1) Comparison to other studies and methods of detection for *C. pecorum*

One important question arising from this study is how prevalence estimates based on DNA isolated from scats compare to those from other commonly used methods of detection. Previous studies of *Chlamydia* in the Raymond Island koala population have reported varying values of prevalence, ranging from 86% (43/50) in 1985 (Mitchell *et al.* 1988) and 71% (73/103) in the early 1990s (Emmins 1996) to 33% (50/153) in 2010–2015 (Legione *et al.* 2016b; Table A1). The prevalence of infection for the Raymond Island koala population found in this study was 81% (21/26), which is similar to the results reported by Mitchell *et al.* (1988) and Emmins (1996), but different ($p < 0.05$) from those reported by Legione *et al.* (2016b) (Table A1). This difference may be the result of the method of detection used by each study. Mitchell *et al.* (1988) and Emmins (1996) detected *Chlamydia* antibodies in blood samples, Legione *et al.* (2016b) used PCR detection of *Chlamydia* DNA in urogenital tract (UGT) swab samples, while this study used PCR detection of *C. pecorum* DNA in DNA isolated from scats.

Emmins (1996) found an all or nothing trend when detecting serum *Chlamydia* antibodies by ELISA, which made the classification of positive and negative animals unequivocal. The interpretation was that, once infected, animals never became completely free of infection, with persistent low levels of infection continuing to promote the production of antibodies. Such infections could persist in the GIT of koalas (Rank & Yeruva 2014) and may be a source of future reinfection of the UGT, for example during mating. Both antibody ELISA and DNA

isolation from scats, may thus be detecting previous UGT infections that have since resolved but are persisting in the GIT; in contrast, qPCR of UGT swab samples may detect active UGT infection only. This may explain why the results of the current study are similar to those reported by Mitchell *et al.* (1988) and Emmins (1996) using serological methods, and higher than those reported by Legione *et al.* (2016b) using PCR of UGT swab DNA. The differences in prevalence rate between this study and the Legione *et al.* (2016b) study potentially provides tentative support for the GIT as a reservoir of *C. pecorum* infection in koalas as it is in other hosts (Burach *et al.* 2014; Rank & Yeruva 2014).

Table A1 Comparison of results from this study, which used DNA isolated from scats to detect *C. pecorum*, with results from Mitchell *et al.* (1988) and Emmins (1996) who determined the prevalence of *Chlamydia* using serological tests and Legione *et al.* (2016b) who used real time PCR to detect *C. pecorum* in UGT swabs.

Reference	Method	SG	RI	OTW	FI
Mitchell <i>et al.</i> (1988)	Serological	NA	86% (43/50)	NA	NA
Emmins (1996)	Serological	62% (26/42)	71% (73/103)	NA	4% (8/190)
Legione <i>et al.</i> (2016b)	DNA from UGT swabs (qPCR)	37% (11/30)¹ B (1), C (3), F (3), M (1)	33% (50/153) B (49)	7.2% (15/210) B (2), L (9)	0.84% (2/237) N (2)
<i>This study</i>	DNA isolated from scats (qPCR)	61% (107/176) B (9), C (3), F (18), I (1), M (9), O (1)	81% (21/26) B (12)	4.9% (2/41) L (2)	NA

SG: South Gippsland, **RI:** Raymond Island, **OTW:** Cape Otway, **FI:** French Island.

1: Legione *et al.* (2016b) collected samples more broadly across Gippsland

2) Infection rates differed for females and males

Data from amplification of sexing markers ($n=166$) indicated that 69 females and 97 males were sampled in the South Gippsland study area. Comparison of the South Gippsland prevalence data according to gender showed that *C. pecorum* was detected more often ($p=0.01$) in females (74% positive) than males (55% positive), with no significant difference between high and low density areas. The pattern was similar on Raymond Island with 86% (18/21) positive females and 60% (3/5) positive males, however the number of males sampled at Raymond Island was comparatively small so the difference not significant. The presence of *C. pecorum* was similar for females (6/13, 46%) and males (4/9, 44%) in the wildlife shelter group. Differences in rates of infection between females and males were not analysed for remaining populations, all of which had fewer than six individuals that were positive for *C. pecorum* (i.e. QLD, NSW, Mallacoota and Cape Otway).

In this study we found that females were more likely to be positive for *C. pecorum* than males. The results of other studies have been variable. In several studies of koala populations in Queensland, equal rates of chlamydial infection were found between the sexes (Weigler *et al.* 1988; White & Timms 1994; Jackson *et al.* 1999). Another study found male koalas to be 2.7 times more likely to be infected with *C. pecorum* compared to female koalas (Legione *et al.* 2016b). Although sample sizes were small, Weigler *et al.* (1988) found a much higher proportion of sub adult females (5/7) were infected with UGT *Chlamydia* compared to sub adult males (0/3). Age classes of individuals sampled in this study were not known, so differences between age groups may be a factor in the prevalence difference between females and males reported here. The greater number of females testing positive for *C. pecorum* in this study could also reflect behavioural or ecological differences of the koala population in South Gippsland or, alternatively, anatomical differences between the sexes resulting in differential sensitivity of the method used to detect *C. pecorum*. Further investigations regarding

detection of *Chlamydia* in DNA sourced from female and male scats are needed to clarify this.

3) Spatial clustering of *ompA* genotypes in the Strzelecki Ranges

The three major *ompA* genotype groups (B, F and M) detected in the Strzelecki Ranges region were spatially clustered between nine regions (OA1–OA9; Fig. A1). Each *ompA* genotype (B, F or M) was represented three times in the region (Fig. A1). Some spatial *ompA* groups (e.g. OA1 and OA2) corresponded to habitat patches separated by agricultural land, while others (e.g. OA3, OA4 and OA5) were located within continuous adjacent habitat. Population structure could span multiple *ompA* regions and *ompA* regions could contain multiple population clusters (Fig. A1).

The spatial distribution of *C. pecorum* infection with different *ompA* genotypes in the South Gippsland koala population was not random, with different genotypes generally clustering in well-defined groups. Interestingly, however, the spatial arrangement of pathogen *ompA* genotypes did not always align with the population structure of the koala hosts in the area (Fig. A1). Gene flow was identified between areas where different *ompA* genotypes appeared to dominate. This finding is similar to that described in Higgins *et al.* (2012) where variants of the *C. pecorum ompA* F genotype were found in areas separated by landscape features despite evidence of koala movement between regions. This may result from movement of predominantly uninfected individuals (e.g. juveniles), as suggested by Higgins *et al.* (2012). Spatial areas where a single *ompA* genotype dominates might therefore represent overlapping koala home ranges, occupied by a mature group of koalas infected with *C. pecorum* of a particular *ompA* genotype. Whether juveniles settle in their natal home range or disperse to a neighbouring area would therefore dictate the chlamydial strain they would be most likely to contract. If infections can be transmitted via the faecal oral route, however, juveniles with

infected mothers might be expected to first come into contact with *C. pecorum* at around five to eight months of age, when pap is fed to the joey in order to inoculate the digestive system with the microorganisms required to digest eucalypt leaves (Martin & Handasyde 1999).

Incidentally, infection of immature koalas was noted during this study. It is also possible that juveniles infected with the predominant strain of the natal home range may disperse to a region where a different strain dominates, with the new or more pathogenic strain potentially becoming the dominant infection after contact.

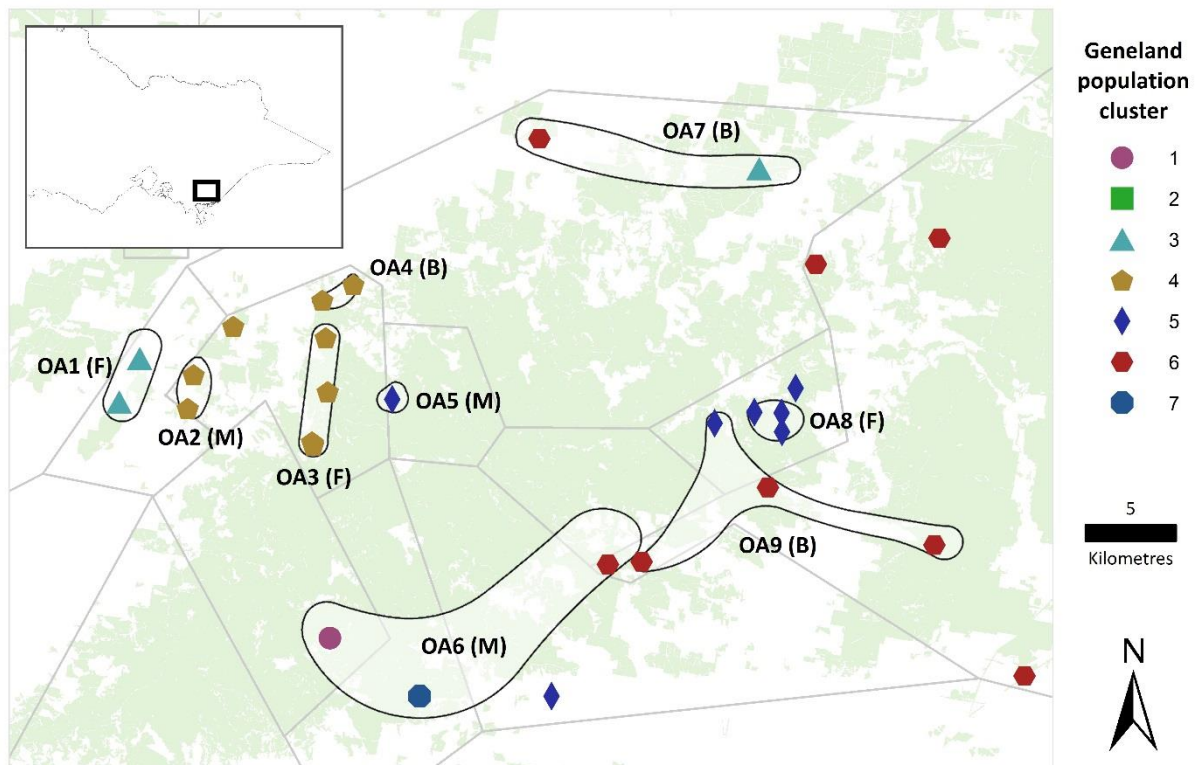


Fig. A1 The spatial distribution of *C. pecorum ompA* genotypes and koala population clusters (as inferred by GENELAND) in the Strzelecki Ranges bioregion. Polygons show approximate regions covered by population clusters. Only individuals with *ompA* genotype data and used in the GENELAND analysis are shown. Green shading indicates the distribution of dense tree cover. GENELAND inferred seven koala genetic clusters, which are indicated by the different symbols listed in the legend. The clustering of chlamydial *ompA* genotypes is shown by the areas outlined in dark grey and labelled *ompA* spatial region one (OA1) through to *ompA* spatial region nine (OA9). The letter following the *ompA* region indicates the *ompA* genotype detected in that area.

4) Phylogenetic analysis of *ompA* genotypes

C. pecorum ompA DNA sequences obtained were aligned using MEGA6 (Tamura *et al.* 2013) alongside koala *C. pecorum ompA* sequences reported by Kollipara *et al.* (2013) and Legione *et al.* (2016b) (Table A2) and exported in fasta format. DNA sequences were converted to protein sequences using the *seqinr* package (Charif & Lobry 2007) in R (R Core Team 2014). Phylogenetic trees were produced by the R package *ape* (Paradis *et al.* 2004) using 5000 bootstrap iterations.

Work carried out by Mohamad *et al.* (2014) previously found a correlation between the virulence of *C. pecorum* strains and the degree of divergence from a putative ancestor at three *C. pecorum* loci (*ompA*, *incA* and ORF663), indicating that *C. pecorum* may become less virulent the further it evolves from the ancestral strain. In order to explore potential differences in strain pathogenicity we used MEGA6 as described by Mohamad *et al.* (2014) to infer an ancestral *ompA* sequence, located at the point at which one representative of each of the eight other *Chlamydia* species joined the phylogenetic tree. MEGA6 was used to calculate genetic divergence between the putative ancestral *ompA* and fifteen koala *ompA* genotypes (A–O), including one novel genotype identified in this study.

Relationships between *C. pecorum ompA* genotypes detected in this study and those previously reported by Kollipara *et al.* (2013) and Legione *et al.* (2016b) are shown in Fig. A2. Four main clades were evident. Clades one (C, G, I and M) and four (E, F and N) included genotypes that have been detected in northern (QLD, NSW) and southern (VIC) koala populations. Clade three was made up of genotypes detected in northern koala populations only (A, H, J and K), while clade two contained genotypes detected in southern koala populations only (B, L, O). Interestingly, the three main *ompA* genotypes detected in the

Strzelecki Ranges bioregion were divergent from one another, each being from a different clade (Fig. A1; B: clade 2, F: clade 4 and M: clade 1).

Evolutionary distance from the putative *ompA* ancestor ranged from 7.2% to 13.4% (Fig. A3). Genotypes E, F and N were the most divergent (13%) followed by genotypes L (11%) and B (10%), while *C. pecorum* genotypes A, C, G, H, I, J, K, M and O had all diverged less than 9% from the putative ancestral *ompA*.

Based on Mohamad *et al.*'s (2014) finding that less pathogenic *C. pecorum* strains are likely to be more divergent, genotypes E, F, N, L and B are, potentially, less pathogenic than strains A, C, G, H, I, J, K, M and O. Interestingly, in this study, five of the six least divergent *ompA* genotypes were found in Queensland and/or New South Wales koala populations (Fig. A3).

The most commonly detected South Gippsland genotype group (F) was relatively more divergent than other genotypes found in the region. If genotype F is less pathogenic than others, this could explain the apparent lack of severe disease in the region. However, genotype F is also common in the northern states, so can presumably cause disease symptoms, although genotype F variants do differ between northern and southern regions. Lower divergence in South Gippsland genotypes C, I, M and O may indicate these strains have a greater pathogenic potential compared to genotypes B and F, but further investigation of this hypothesis is needed. Mohamad *et al.* (2014) used three genes (*ompA*, *incA* and ORF663) to predict *C. pecorum* virulence; further studies involving all three genes may increase the strength of such analyses and provide further insight into genetic differences between *C. pecorum* strains infecting koalas in the South Gippsland region.

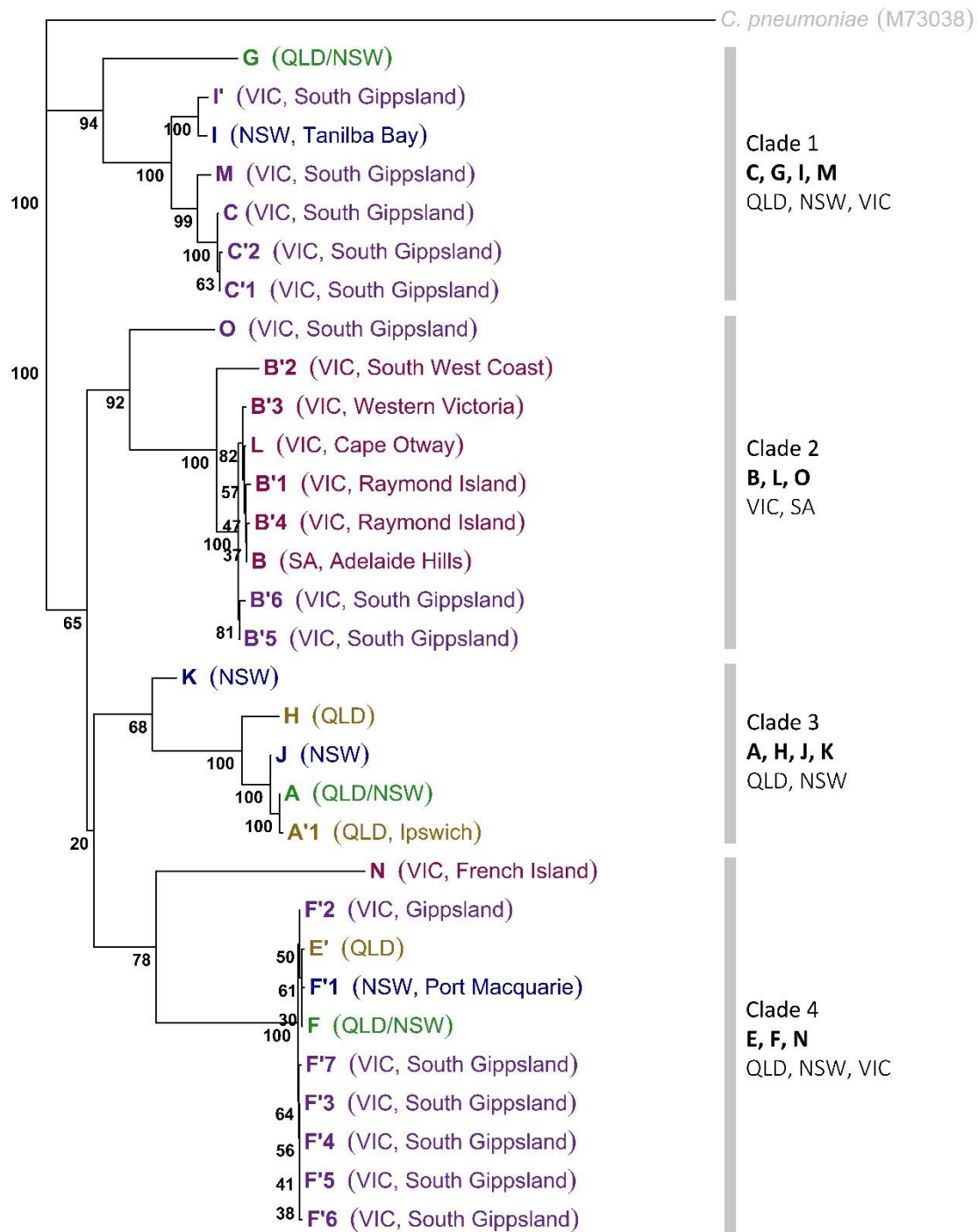


Figure A2 Phylogram of the *C. pecorum ompA* genotypes detected in this study and by Kollipara *et al.* (2013) and Legione *et al.* (2016b), rooted to *Chlamydia pneumoniae* (M73038). Locations at which genotypes have been detected to date are listed in parentheses. QLD: Queensland; NSW: New South Wales; VIC: Victoria.

Table A2 List of *C. pecorum* ompA genotypes described to date in koalas. The origins of the koalas in which the genotype was detected are also listed along with the GenBank accession number where available.

ompA genotype	Locations detected	GenBank accession number/s	Reference/s
A	QLD: Mutdapilly, Redland Bay, Lone Pine Koala Sanctuary, Currumbin Sanctuary. NSW: Tanilba Bay	KF150132	Jackson <i>et al.</i> (1997); Kollipara <i>et al.</i> (2013)
A'Ko1 ^{*1}	QLD: Southeast region. NSW: Gunnedah	JF309281	Higgins <i>et al.</i> (2012)
A'1	QLD	KY913821	This study
B	SA : Adelaide Hills	KF150133	Kollipara <i>et al.</i> (2013)
B'1	VIC: Raymond Island, Healesville, Strathbogie	JF309282 ; KU214248; KY913822	Jackson <i>et al.</i> (1997); Higgins <i>et al.</i> (2012); Legione <i>et al.</i> (2016b); this study ^{*2}
B'2	VIC : South West Coast	KU214249	Legione <i>et al.</i> (2016b)
B'3	VIC : Western Victoria	KU214251	Legione <i>et al.</i> (2016b)
B'4	VIC: South Gippsland	KY913823	This study
B'5	VIC: South Gippsland	KY913824	This study
B'6	VIC: South Gippsland	KY913825	This study
C	VIC: koala originally from Victoria but located at Featherdale Wildlife Park, NSW; Greater Gippsland; KCC	KU214245	Jackson <i>et al.</i> (1997); Legione <i>et al.</i> (2016b); this study
C'Ko3	VIC: Strathbogie	JF309283	Higgins <i>et al.</i> (2012)
C'1	VIC: South Gippsland	KY913826	This study
C'2	VIC: South Gippsland	KY913827	This study
D	Australia	NA	Jackson <i>et al.</i> (1997)
E	NSW: Emerald, Port Macquarie, Featherdale Wildlife Park	NA	Jackson <i>et al.</i> (1997)
E'	QLD: Narangba, Lower Beechmont	KF15013	Kollipara <i>et al.</i> (2013)
F	QLD: St Bees Island, Brendale, North Stradbroke Island, East Coomera, Lower Beechmont, Elanora. NSW: Byron Bay, Port Macquarie, Tanilba Bay	KF150135; KY913828	Kollipara <i>et al.</i> (2013); this study
F'1	NSW: Port Macquarie	KF150136	Kollipara <i>et al.</i> (2013)
F'2	VIC: Greater Gippsland	KU214246	Legione <i>et al.</i> (2016b)

ompA genotype	Locations detected	GenBank accession number	Reference/s
F'3	VIC: South Gippsland	KY913829	This study
F'4	VIC: South Gippsland	KY913830	This study
F'5	VIC: South Gippsland	KY913831	This study
F'6	VIC: South Gippsland	KY913832	This study
F'7	VIC: South Gippsland	KY913833	This study
F'Ko5a	QLD: Southeast region. NSW: Port Macquarie, Anna Bay, Lismore	JF309285	Higgins <i>et al.</i> (2012)
F'Ko5b	NSW: Port Macquarie	JF309286	Higgins <i>et al.</i> (2012) ^{*2}
F'Ko5c	NSW: Port Macquarie	JF309288	Higgins <i>et al.</i> (2012)
F'Ko5d	NSW: Port Macquarie	JF309289	Higgins <i>et al.</i> (2012)
F'Ko5e	QLD: Southeast region	JF309290	Higgins <i>et al.</i> (2012)
F'Ko5f	NSW: Port Macquarie	JF309291	Higgins <i>et al.</i> (2012)
F'Ko5fi	NSW: Port Macquarie	JF309292	Higgins <i>et al.</i> (2012)
F'Ko5g	NSW: Port Macquarie	JF309293	Higgins <i>et al.</i> (2012)
G	QLD: Southeast region, Brendale, East Coomera. NSW: Tanilba Bay. SA: Adelaide Hills	JF309284; KF150137	Higgins <i>et al.</i> (2012); Kollipara <i>et al.</i> (2013)
H	QLD: East Coomera, Lower Beechmont	KF150138	Kollipara <i>et al.</i> (2013)
I	NSW: Tanilba Bay	KF150139	Kollipara <i>et al.</i> (2013)
I'	VIC: South Gippsland	KY913834	This study
J	NSW: Port Macquarie, Tanilba Bay	KF150140	Kollipara <i>et al.</i> (2013)
K	NSW: Tanilba Bay	KF150141	Kollipara <i>et al.</i> (2013)
L	VIC: Cape Otway National Park	KU214250; KY913835	Legione <i>et al.</i> (2016b); this study
M	VIC: Greater Gippsland, South Gippsland	KU214247; KY913836	Legione <i>et al.</i> (2016b); this study
N	VIC: French Island	KU214244	(Legione <i>et al.</i> 2016a); Legione <i>et al.</i> (2016b)
O	VIC: South Gippsland, Koala Conservation Centre	KY913837	This study

The approximate number of base pairs in the *C. pecorum ompA* gene utilised in each study were: Jackson *et al.* (1997), 400 bp; Higgins *et al.* (2012), 700 bp; Kollipara *et al.* (2013), approximately 1120 bp; Legione *et al.* (2016b), 1170 bp and for this study, 1050 bp. Due to differences in the size of the *ompA* fragment analysed by the different studies, some genotypes based on a smaller portion of the *ompA* gene may not be able to be differentiated from genotypes based on a longer portion of the gene.

*1: A'Ko1 may be equivalent to genotypes A or J

*2: F'Ko5a may be equivalent to E'2, F, F'1 or F'2

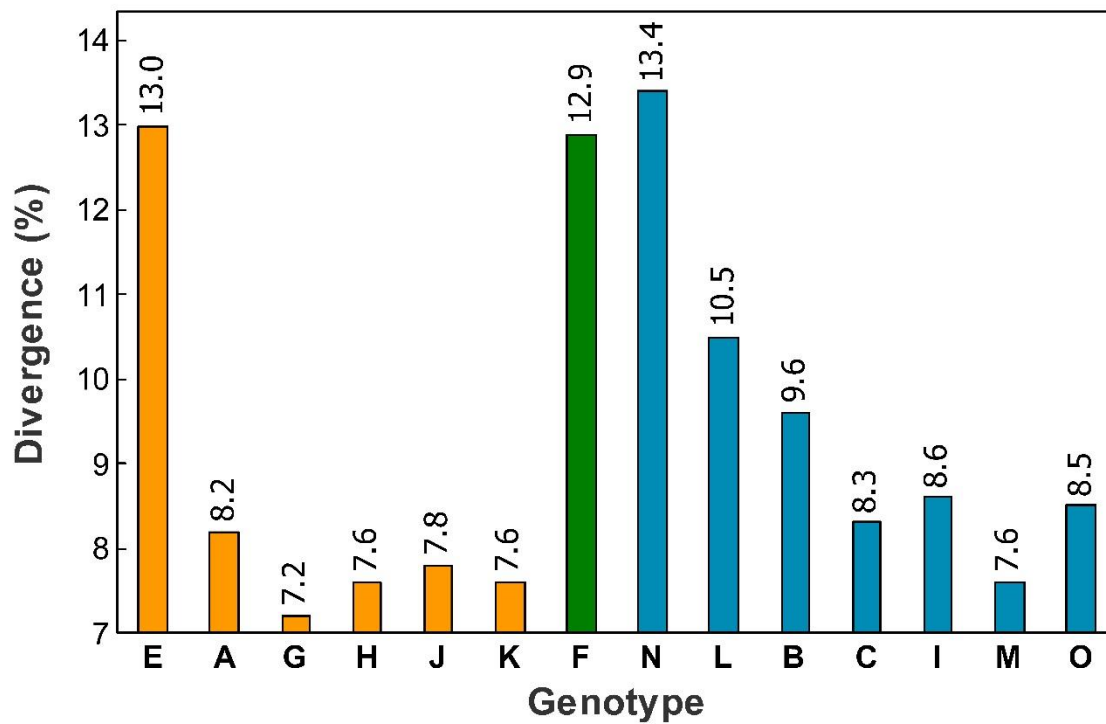


Figure A3 Evolutionary divergence of koala *ompA* genotypes from a putative *ompA* ancestor. Genotypes detected in northern populations in Queensland and/or New South Wales are shaded orange (E, A, G, H, J and K), genotypes found across the koalas range (in Queensland, New South Wales and Victoria) are shaded green (F) and genotypes detected in southern (Victorian and South Australian) populations only are shaded blue (N, L, B, C, I, M and O).

KoRV

5) KoRV-A *env* genotypes

Two genetic variants of KoRV-A were detected in South Gippsland, KV01 and KV03. Analysis of the small number of samples for which both KoRV-A genotypes and koala mtDNA haplotypes were available revealed that the two koalas with mtDNA haplotype Pc17 were positive for KV01 and the six individuals with koala mtDNA haplotype Pc27 were positive for KV03. This may suggest that Pc17 and Pc27 haplotype koalas entered Victoria at different points in history, potentially via two separate historical population expansions or, alternatively, by past human mediated movement of koalas; the distribution of mtDNA haplotype diversity prior to European settlement is unknown. A more expansive survey of KoRV-A *env* genotypes present in South Gippsland is needed to determine whether KV01 is found exclusively in Victorian koalas with the Pc17 mtDNA control region haplotype. It would also be of interest to compare KoRV-A copy number between *env* genotypes to determine whether KV01 may be endogenous in Victorian koalas (with copy number ≥ 1 KoRV-A copy per cell) and KV03 exogenous in Victorian koalas (with copy number ≤ 1 KoRV-A copy per cell); this may account for the range of copy numbers observed in other studies (Simmons *et al.* 2012; Legione *et al.* 2017).

6) KoRV-A infection and population density

Population density did not appear to influence the prevalence of KoRV-A in South Gippsland, which may suggest that exogenous KoRV-A is not easily transmitted between individuals. This is supported by the finding that KoRV-A infection rates in Victorian koalas have remained stable over a period of about three years, with KoRV-A prevalence on French and Raymond Islands of around 20 – 30% (Table A3; Simmons *et al.* 2012; Legione *et al.* 2017; this study). In contrast, on Kangaroo Island (South Australia), the koala population is

overabundant, and the rate of infection has been observed to increase from 0% ($n=26$) in 2004, to 15% ($n=162$) in 2007 and 36% ($n=50$) in 2009 (Simmons *et al.* 2012). French and Raymond Island populations, however, both also have relatively high koala densities. If the virus was easily transmitted between individuals, increasing rates of KoRV-A infection might also be expected in these populations. However, this does not appear to be the case. An alternative explanation for observed differences in transmissibility between populations, could be environmental factors or varying pathogenicity of KoRV-A strains infecting different populations. KoRV-A *env* genotypes for Kangaroo Island koalas have not been reported; further genetic investigations may provide a greater understanding of these differences.

Table A3 Comparison of KoRV-A prevalence in DNA isolated from scats with results reported by Simmons *et al.* (2012).

Population	Simmons <i>et al.</i> (2012)	Legione <i>et al.</i> (2016)	This study
North east NSW	43/43 (100%)	-	17/17 (100%)
French Island (FI)	6/28 (21%)	23/94 (24%)	-
Cape Otway (FI origin)	-	31/178 (17%) ^A	2/11 (18%)
Phillip Island	0/11 (0%)	-	0/16 (0%) [*]
Raymond Island	10/29 (34%)	38/136 (28%)	4/18 (22%)
South Gippsland	18/26 (69%)	6/33 (18%) ^B	39/142 (27%)

^{*} Samples from the Koala Conservation Centre, Phillip Island, where individuals are from a variety of locations including Phillip Island, Brisbane Ranges, Strathbogie and South Gippsland. **A:** Samples were obtained more broadly than Cape Otway alone, throughout the South West region of Victoria. **B:** Samples were collected across all of Gippsland, rather than South Gippsland alone.

Questions for further research

Chlamydia pecorum infection in koalas

- 1) Can *C. pecorum* be transmitted via the faecal-oral route in koalas as in other animal hosts?
- 2) Is the gastrointestinal tract a major site of *C. pecorum* infection in koalas as it is in other animal hosts?
 - a) Could gastrointestinal infection with *C. pecorum* be the source of urogenital infections?
 - b) If so, could procedures such as urogenital swabbing carry a risk of mechanical transfer of *C. pecorum* from the gastrointestinal tract to the urogenital tract?
- 3) Are there differences in *C. pecorum* strain pathogenicity as there are in other animal hosts?
 - a) Do all strains have similar impacts on reproduction rates?

KoRV infection in koalas

- 4) In Victoria, is KoRV-A *env* genotype KV01 found only in koalas with mtDNA control region haplotype Pc17?
- 5) Could *env* genotype variants explain differences in KoRV-A copy number seen in Victorian koalas (e.g. more than one KoRV-A copy per cell or much less than one KoRV-A copy per cell)

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Chapter 10

Discussion





Discussion

The koala is an endemic Australian marsupial whose habitat has been substantially altered since the time of European settlement in 1788 (Bradshaw 2012). Koalas were on the verge of extinction in the early 1900s (Lewis 1954) but some populations recovered and the species was generally considered secure prior to the 1990s. However, due to widespread population declines since the 1990s, koalas are now listed as threatened in the north of their range (Queensland, New South Wales and the Australian Capital Territory; EaCRC 2012). Southern koala populations in Victoria and South Australia are not listed, in part due to differing circumstances between southern koala populations, where some are actively managed due to overpopulation, some appear to be in decline and, for many populations, data are not available (Menkhorst 2004; Menkhorst 2008; EaCRC 2011a, b).

DNA can provide data relevant to the conservation and management of wildlife including information relating population structure, genetic diversity, relatedness, inbreeding, gene flow and rates of migration (Frankham 2003; DeSalle & Amato 2004). DNA sampling of koalas can be difficult as they often reside in the tops of Eucalypt trees, many of which may be well over 30 metres tall. Sampling difficulty is also increased where koala population density is low and/or where terrain limits accessibility. As population densities, vegetation and terrain varies widely across the koala's range, some populations may be more difficult to sample using animal capture, which may result in a lack of data for some regions. In addition to this, the need to capture animals can impose time and cost constraints, thereby limiting the number of samples obtained. DNA isolated from scats provides a novel approach for obtaining koala population data. A wealth of information, critical for evidence-based koala management may be obtained from DNA (e.g. population structure and fragmentation, genetic diversity and gene flow). The availability of non-invasive methods to sample koala DNA will therefore be

an invaluable tool for koala conservation, facilitating more, and more evenly distributed sampling across the koala's range.

DNA obtained using the non-invasive sampling methods described in this thesis (summarised in Fig. 1), provide reliable multi locus microsatellite genotypes, mtDNA sequences and the gender of the individual sampled, generating data that can be used confidently for population, conservation and landscape genetics. In addition to providing genetic data relating to koalas, this thesis also demonstrates that genetic material sourced from scats includes the DNA of two pathogenic organisms, *Chlamydia pecorum* and koala retrovirus (KoRV). DNA from these organisms can be isolated and amplified, providing prevalence data and facilitating genetic studies of these pathogens.

The techniques presented in this thesis can accelerate the collection of both koala population data and pathogen (*C. pecorum* and KoRV) prevalence data. Such techniques facilitate the study of large numbers of wild koala populations, including those at low density, thereby offering a more complete picture of koala conservation. The power of this sampling approach is demonstrated in the thesis, which describes the results of a genetic study based on koala scats collected from more than 350 individual koalas from the south-eastern state of Victoria. These samples were obtained in a relatively short time span with little cost associated with fieldwork and provided data regarding the genetic structure and prevalence of infection with *C. pecorum* and KoRV for Victorian koala populations.

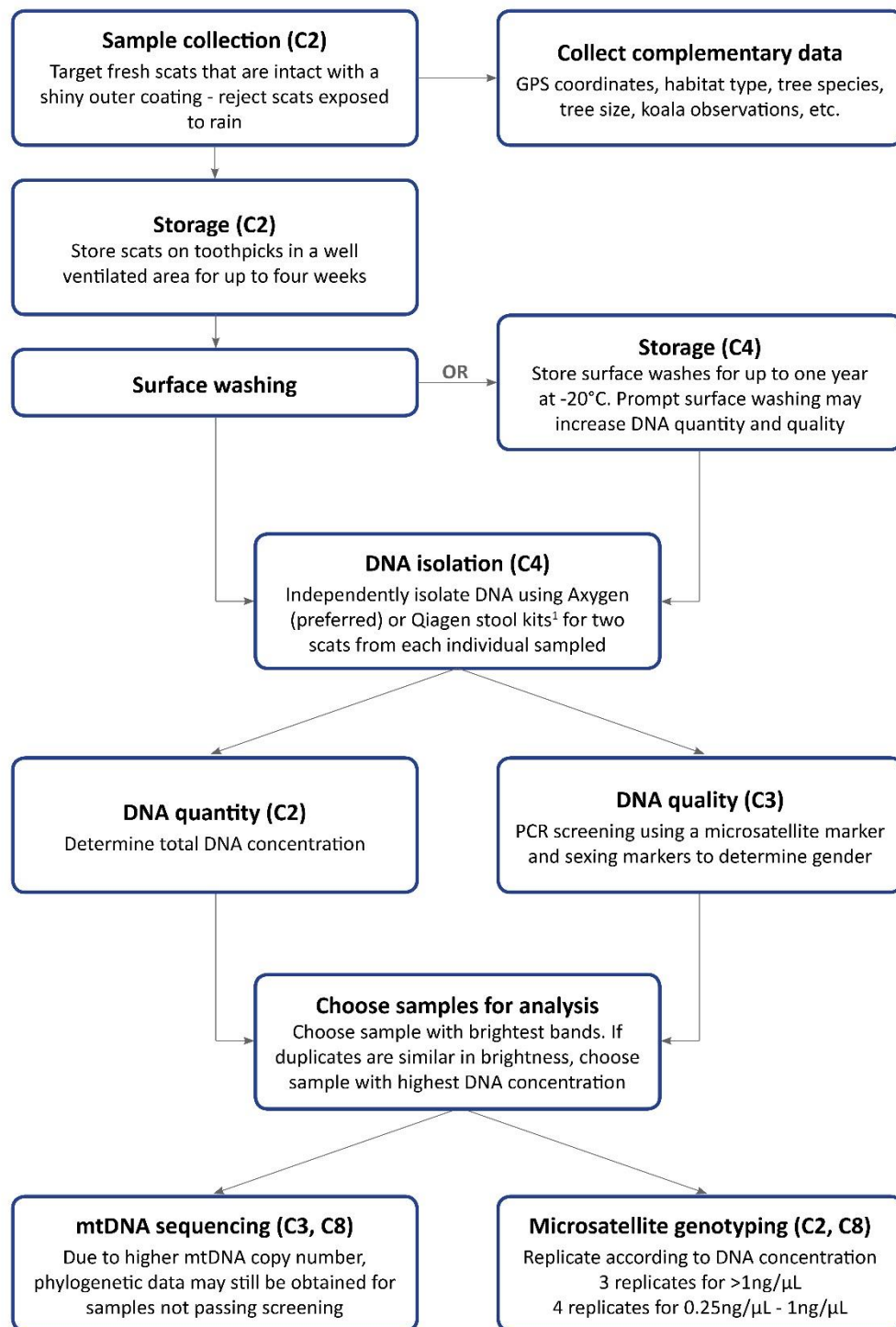


Figure 1 a) Flowchart of the general methodology used in this study for data collection: sample collection, DNA isolation and screening for DNA quantity and quality. References to the relevant chapters within this thesis are indicated within parentheses following headings (e.g. C2: chapter 2).

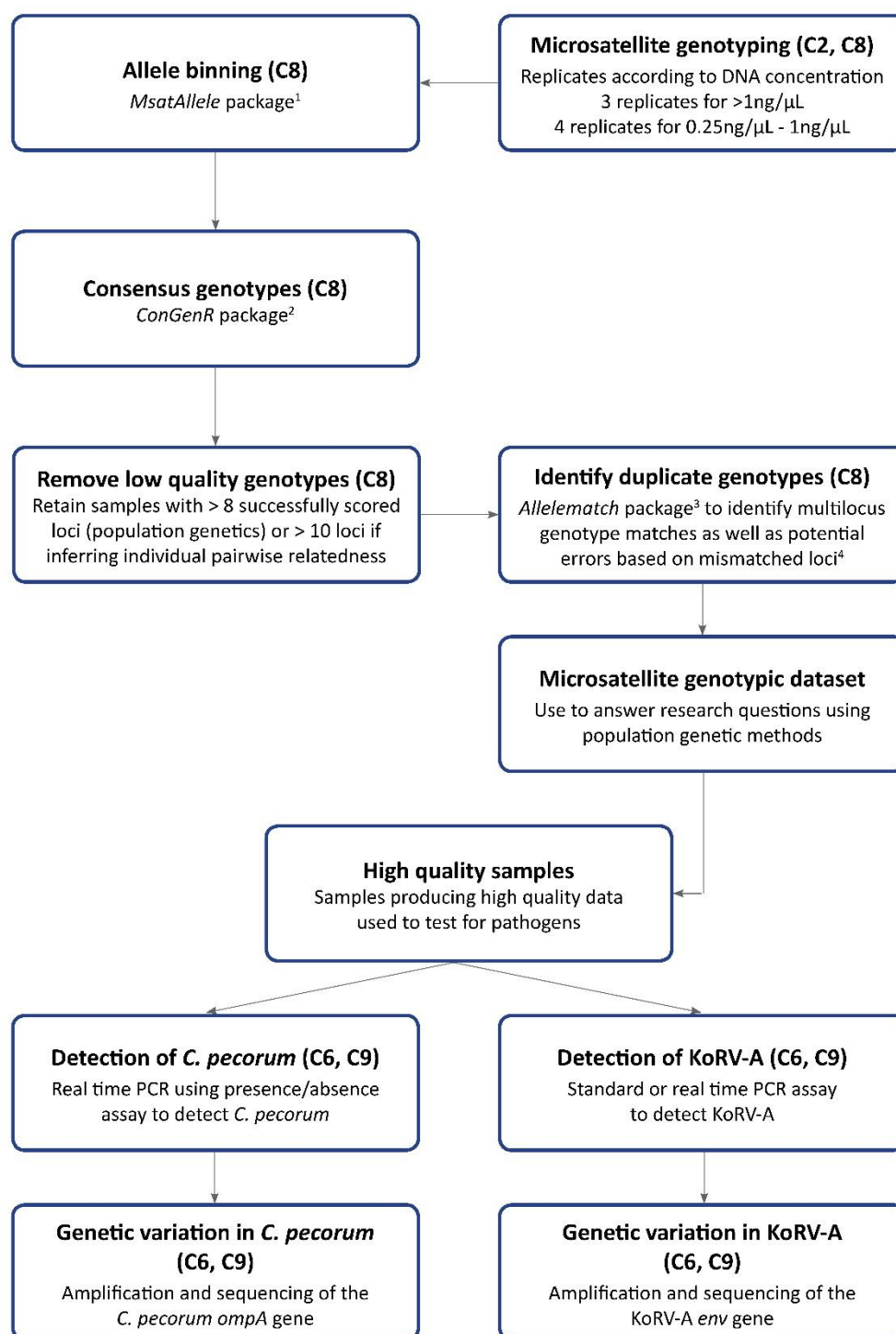


Figure 1 b) Flowchart of the general methodology used in this study for data collection: treatment of genotype data to minimise errors and screening of pathogens.

¹ Alberto (2009); ² Lonsinger and Waits (2015). References to the relevant chapters within this thesis are indicated within parentheses following headings (e.g. C2: chapter 2).

Value of a non-invasive method for koala research

Non-invasive sampling methods have the potential to change the way in which koala populations are routinely studied, providing relatively large sample sizes and robust population datasets while at the same time minimising any negative impacts involved with animal capture and handling. DNA isolated from scats can identify isolated populations and provide estimates of inbreeding. Isolation and inbreeding are key genetic factors in the extinction process (Frankham 2005; Frankham *et al.* 2012). Elimination of potential risks associated with animal capture and invasive procedures is also likely to be of high priority for studies involving declining populations. The collection of scat samples for DNA analysis may be also be expedited by use of finding aids such as scat detection dogs (Cristescu *et al.* 2015).

There is currently a high level of concern for koala populations throughout their range (EaCRC 2011a). Coupling the collection of scats for DNA analysis with koala surveys utilising scats to determine the presence and density of koalas (e.g. Sullivan *et al.* 2004; Phillips & Callaghan 2011) would also be useful, thereby providing genetic data and complementing survey data, which could subsequently be used to examine landscape factors impeding or facilitating gene flow (Storfer *et al.* 2006).

Historic gene flow

Koala populations from South East Queensland to Victoria were found to exhibit a strong pattern of isolation by distance (chapter 8). This pattern indicates that, historically, koala populations across their range are likely to have been connected by low levels of gene flow (e.g. Fig. 2a) that may no longer exist for many populations due to numerous population extirpations occurring in the past (Fig. 2b).

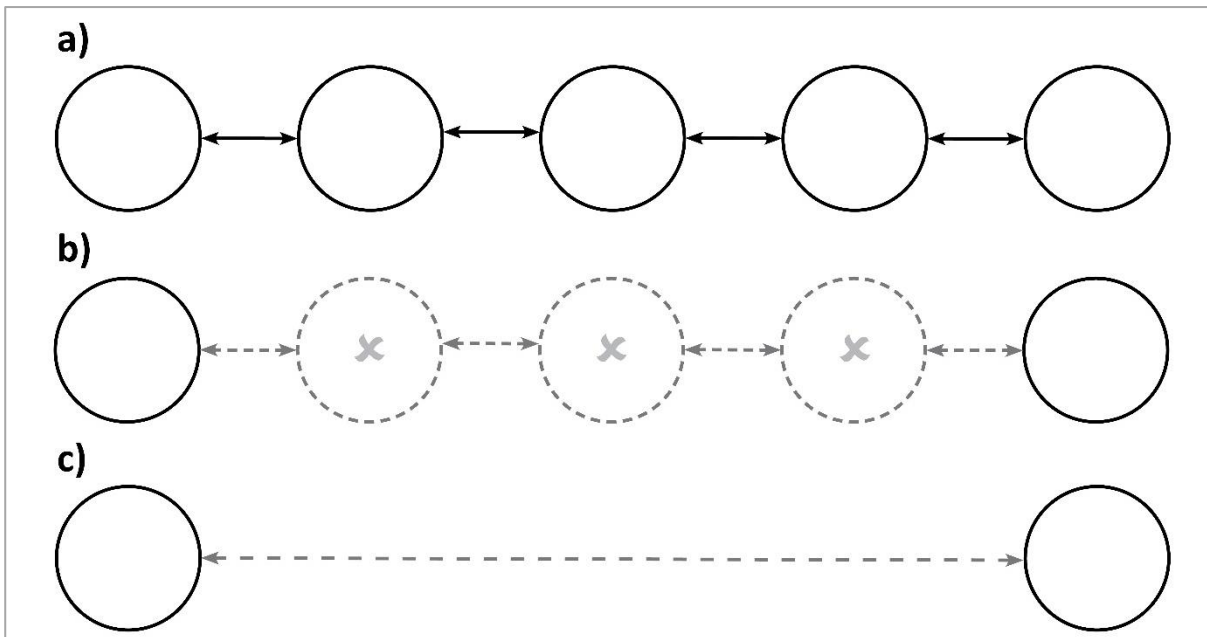


Figure 2 (a) Strong isolation by distance (chapter 8) indicates that koala populations are likely to have been historically connected by low levels of gene flow as illustrated. (b) Widespread land clearing, hunting for the fur trade and forest fires after European colonisation has resulted in the extirpation of many koala populations. (c) The result is the isolation of populations between which migration may no longer be possible due to distance and/or barriers to movement.

Re-establishing gene flow between isolated populations, even at low levels, can have beneficial effects for genetic diversity and adaptive potential (Vila *et al.* 2003; Frankham *et al.* 2012). Artificial movements of low numbers of individuals between isolated populations may be a useful conservation measure in some cases (Fig. 2c; Aitken & Whitlock 2013; Frankham 2016). Migration between extant koala populations that have hypothetically become genetically isolated separated by a large distance (as illustrated in Fig. 2c) is not likely to have occurred directly in the past, but rather by low levels of migration between intermediate populations separated by shorter distances (Fig. 2a).

Our genetic data indicate a potential phylogenetic divide between koalas sampled in Queensland and those south of Port Macquarie (northern New South Wales). Other research has also suggested such a divide (Houlden *et al.* 1999; Ruiz-Rodriguez *et al.* 2016) although, overall, genetic studies currently support the presence of a single species (Houlden *et al.* 1999; Neaves *et al.* 2016). However, the results of these studies have been based on one hypervariable region of the mitochondrial genome (the control region). Further investigations using additional genetic markers and karyotyping are therefore warranted to determine whether Queensland and more southern koalas in New South Wales, Victoria and South Australia, constitute separate evolutionary significant units or subspecies. This will be important information for the purpose of assessing the viability of assisted gene flow between particular populations as a future conservation action.

Risks associated with relocation for conservation purposes

Reintroductions, genetic rescue and assisted gene flow have the potential to provide significant conservation benefits. When crossing populations there are, however, a range of factors (Hedrick 2005) and risks that require consideration (Weeks *et al.* 2011). A framework for assessing the benefits and risks of proposed translocations are provided in Weeks *et al.* (2011) and potential risks are outlined below.

Outbreeding depression

Outbreeding depression occurs when a cross between populations results in reduced reproductive fitness in hybrids and their offspring and is most common when different species or sub-species are crossed (Frankham *et al.* 2012). Long term population isolation and adaptations to local habitats may increase the risk of outbreeding depression (Frankham *et al.* 2011). The likelihood that outbreeding depression will occur can be assessed (Frankham *et al.*

2011; Weeks *et al.* 2011) but requires an understanding of historical and contemporary population structure.

Loss of genetic individuality and genetic homogenisation

Historically separate populations are likely to have unique genetic compositions, that are important to conserve, but may be eliminated by transfer of alleles from the translocated population (Hedrick 2005). Swamping of functional genetic diversity in a resident population may result in decreased fitness for the local environment (Frankham *et al.* 2011; Weeks *et al.* 2011). Facilitating gene flow between populations not connected directly in the past may also result in the loss of rare alleles and homogenisation of genetic diversity across large areas (Olden *et al.* 2004). A lack of within population variation may negatively impact the ability of a species to expand into new environments or adapt to future environmental changes (Olden *et al.* 2004).

Mortality of translocated individuals

Increased mortality is also associated with translocation. One study found that 37.5% of translocated koalas did not survive one year post translocation, although reasons for deaths were not identified (Whisson *et al.* 2012). Another study carried out by Santamaria (2002) found a mortality rate of around 24% following translocation. Causes of death in this study were mostly attributed to injury or starvation (Santamaria 2002). High mortality of translocated individuals may be related to habitat quality, habitat patch size, density of the species at the release site, resource competition and disrupted social organisation (Gundersen *et al.* 2002; Santamaria 2002; Short 2009).

Movement of pathogens

Another major consideration is the movement of pathogens (both known and unknown) between populations (Daszak *et al.* 2000), which will be of particular concern when the pathogen is new to the recipient population. The possibility that different strains of *C. pecorum* differ in their ability to cause disease in koalas (as it does in other animal hosts) is an important consideration (Mohamad *et al.* 2014), as is our currently limited understanding of KoRV and its pathogenesis. The introduction of novel pathogens to a population may lead to extirpation, especially where a population is already compromised in some way.

A large number of attempted translocations fail (Fischer & Lindenmayer 2000; Short 2009). Decisions to utilise translocations for conservation purposes therefore require careful consideration (Weeks *et al.* 2011). It is paramount that threatening processes causing population decline are identified and rectified before translocations are undertaken. Where the threats to a population stem from a lack of habitat, or poor quality habitat, such conservation efforts will be of little use. Improving habitat size, connectivity and quality are therefore a high priority and vital for the long-term conservation of koala populations. Increasing habitat connectivity will also facilitate natural gene flow between subpopulations, thereby reducing the need for artificial movements of animals and the risks that they entail.

Genetic diversity across the koala's range

The near extinction and re-establishment of koala populations in Victoria and South Australia during the 20th century has resulted in the reduction and homogenisation of genetic diversity within affected southern koala populations. Populations derived from island stock analysed in this study (chapter 8) were found to have an average of 3.3 alleles per locus while the South Gippsland koala population was found to have much greater diversity with an average of 7.2 alleles per locus. The level of genetic diversity present in the South Gippsland koala

population is comparable to populations throughout their range; reported mean alleles per locus have ranged from 3.2 to 10.3 for populations in New South Wales (Lee *et al.* 2010b; Lee *et al.* 2012; Dennison *et al.* 2017) and from 5.9 to 10.2 for mainland populations in south-east Queensland (Houlden *et al.* 1996; Lee *et al.* 2010a). Genetic diversity for island populations in Queensland ranges from 2.5 to 5.7 alleles per locus (Lee *et al.* 2013), which is also comparable to the level of diversity detected in island derived koala populations within Victoria (chapter 8).

Although Victorian koala populations are considered to be much less diverse than populations in New South Wales and Queensland (EaCRC 2011b), island derived populations in Victoria have diversity comparable to northern populations that have been subjected to bottlenecks, such as Campbelltown in NSW and island populations in QLD (Lee *et al.* 2010b; Lee *et al.* 2013). In Victoria, the South Gippsland koala population encompasses genetic diversity significantly greater than island derived populations and within the range reported for studies of more northern populations (Houlden *et al.* 1996; Lee *et al.* 2010a; Lee *et al.* 2012; Lee *et al.* 2013; Dennison *et al.* 2017). Direct comparisons between studies should however be treated cautiously, as differences in the markers used and sampling effort may also affect differences between results.

The importance of the South Gippsland koala population/s

The conservation of the South Gippsland koala population and its genetic diversity is important for three main reasons. The first relates to the significance of the South Gippsland koala population itself. Current members of the South Gippsland koala population are descendants of koalas that survived the population bottleneck of the early 1900s. This thesis demonstrates that the South Gippsland koala population is a remnant population not derived from the translocation of island animals (chapter 8). Most other extant koala populations in

Victoria are believed to be descended from translocated island koalas and are therefore likely to be characterised by decreased genetic diversity. Although evolutionary potential was not assessed here, the greater genetic diversity of the South Gippsland koala population may increase its ability to adapt to and survive future environmental challenges when compared to island derived populations. Protection of the remnant South Gippsland koala population is necessary to conserve its genetic diversity.

Conservation of the South Gippsland koala population is also important for the conservation of all koala populations in Victoria. If the negative effects of low genetic diversity in other populations were to become apparent in the future, ‘genetic rescue’ may be a potential conservation action (Vila *et al.* 2003; Hedrick & Fredrickson 2010). Genetic rescue is a term used to describe the introduction of small numbers of individuals from an outbred population into an inbred population suffering the negative effects of low genetic diversity (Hedrick & Fredrickson 2010). In the case of Victorian koalas, individuals from the South Gippsland population would be good candidates for potential genetic rescue of inbred populations in the future, as koala populations derived from French and Phillip Island stock were founded by Gippsland koalas (Lewis 1934, 1954; Wedrowicz *et al.* 2017), and therefore have similar genealogies. South Gippsland koalas would therefore be ideal candidates for increasing genetic diversity and fitness into island derived populations in decline. Before undertaking such a strategy, however, potential risks such as the transfer of pathogens between populations and potential negative impacts on the outbred population would need to be assessed (Frankham *et al.* 2011; Weeks *et al.* 2011).

The third reason for the conserving and managing the South Gippsland koala population is for the long term conservation of the koala as a species. Climate change is predicted to have significant impacts on koala populations (Adams-Hosking *et al.* 2011a; Adams-Hosking *et al.*

2011b; Adams-Hosking *et al.* 2012). Climate change may impact koalas via altered thermoregulatory requirements (Ellis *et al.* 2010), the contraction and shift of suitable habitat (González-Orozco *et al.* 2016) and differences in the nutritional composition of eucalypt leaves (Gleadow *et al.* 1998). The latter may result in some presently preferred eucalypt species becoming less suitable for koalas in the future (DeGabriel *et al.* 2010). In Victoria, an increased frequency of fire associated with climate change (Hennessy *et al.* 2005) is also likely to impact on the amount of available habitat.

Although koalas are currently considered a single species (Houlden *et al.* 1999), a considerable level of morphological (Menkhorst & Knight 2010; Briscoe *et al.* 2015) and genetic diversity (Chapter 8) exists across their range. Some variation in koala populations is likely to be related to adaptations to local conditions. Changes in climate and habitat suitability are unlikely to occur homogeneously across the koala's range, so it will be difficult to predict the capacity of any one koala population to adapt to future environmental changes (Pauls *et al.* 2013). Populations in the north of the koala's range may become extinct with populations surviving in the south or vice versa. Alternatively, small scattered populations may survive across the koala's range in refugia, which may re-expand when conditions permit. The conservation of as much diversity as possible across the koala's range is therefore highly important.

State and Federal government recommendations addressed

This research has addressed several actions and recommendations from Victoria's Koala Management Strategy (Menkhorst 2004) and the Senate Inquiry into the Status, Health and Sustainability of Australia's Koala population (EaCRC 2011a). Victoria's koala management strategy was designed to assist in achieving the goals of the National Koala Conservation Strategy (since superseded by the National Koala Conservation and Management Strategy

2009-2014) within the state of Victoria. Victoria's koala management strategy outlines 16 objectives and 35 action statements, two of which directed this research. These were action 17, which was to: "initiate a detailed survey of genetic diversity, using microsatellite and mitochondrial DNA markers, across South Gippsland, from Western Port to Sale and from the Princess Highway to Refuge Cove, Wilsons Promontory" and parts of action 22 which was to "initiate a survey of *Chlamydomphila*¹ status of koala populations throughout Victoria", one of the specific aims being to identify "species and strains of *Chlamydomphila* present" (Menkhorst 2004). At a National level, the report of the 2011 Senate Inquiry into the Status, Health and Sustainability of Australia's Koala Population (EaCRC 2011a) recommended "research into the genetic diversity of the koala including a population viability assessment of the southern koala and determining priority areas for conservation nationally".

By sampling a large number of koalas in South Gippsland, this research has addressed action 17 from Victoria's Koala Management Strategy in detail, and has shown that the South Gippsland koala population is unique from, and has greater genetic diversity than, populations derived from French or Phillip Islands. Koalas belonging to the remnant koala population in South Gippsland (i.e. not having an island derived ancestry) were identified as far west as Inverloch and Outtrim and as far east as Loch Sport (Fig. 3), indicating that the remnant koala population may be more widely distributed geographically than previously thought. Further sampling expanding outwards from the area sampled in this study is needed in order to determine how far the remnant population extends. Releases of island derived koalas are prohibited in the South Gippsland region from 146 degrees to 147 degrees of longitude and south of the Princes Highway in order to protect further loss of remnant diversity via dilution or genetic swamping by admixture with island derived individuals (Menkhorst 2004). As

¹ *Chlamydomphila* is equivalent to *Chlamydia*. The difference is the result of past subdivision of the *Chlamydiaceae* family into two genera, *Chlamydia* and *Chlamydomphila* and subsequent reclassification of all species into a single genus, *Chlamydia* (Sachse *et al.* 2015).

shown in Fig. 3, this study found that the geographical boundary of the South Gippsland koala population extends well beyond this zone, which should be enlarged to coincide with the currently identified distribution of the South Gippsland koala population.

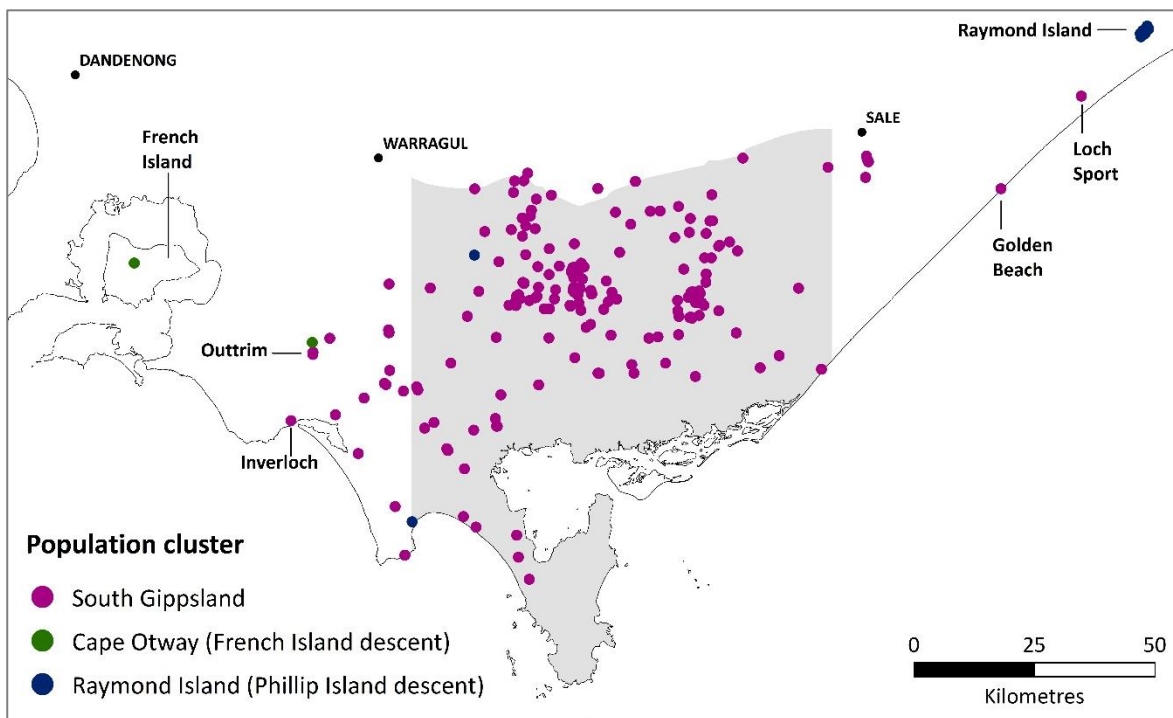


Figure 3 Population clustering of individual koalas sampled in South Gippsland during this study. Grey shading indicates the current release exclusion area, south of the Princes Highway between 146 and 147 longitudinal degrees (Menkhorst 2004).

Potential for legislative protection for the South Gippsland koala population

Legislation for the conservation of threatened species in Victoria is covered by the Flora and Fauna Guarantee Act 1988 (FFG Act). The FFG Act is currently (April, 2017) under review so eligibility criteria or wording may differ once the new version is released. The current eligibility criteria for listing a species under the FFG Act are:

“11 Eligibility for listing

- (1) A taxon or community of flora or fauna is eligible to be listed if it is in a demonstrable state of decline which is likely to result in extinction or if it is significantly prone to future threats which are likely to result in extinction.
- (2) A taxon of flora or fauna which is below the level of sub-species and a community of flora or fauna which is narrowly defined because of its taxonomic composition, environmental conditions or geography is only eligible for listing if in addition to the requirements of subsection (1) there is a special need to conserve it.”

The existing FFG Act therefore provides for populations under the subspecies level to be listed if there is ‘special need to conserve’ that population and it is in decline or is predisposed to extinction from future threats. There is currently no data available regarding population trends in South Gippsland, it is therefore not known whether the South Gippsland koala population is increasing, stable or declining. Gaining greater insights into the population dynamics of the region by determining population trends would be useful. The distribution of age classes in a population may provide information regarding population trends (e.g. Martin 1981), which could potentially be achieved using non-invasive methods for determining age class (e.g. Flasko *et al.* 2017).

There is special need to conserve the South Gippsland koala population as it is a remnant population possessing greater genetic diversity than other Victorian koala populations (chapter 8). There is also significant future threat to koala populations from climate change and its associated impacts, which are predicted to affect koalas in numerous ways, including shifts in habitat distribution (Adams-Hosking *et al.* 2012; González-Orozco *et al.* 2016) and potential changes to the suitability of some eucalypts that koalas browse (Gleadow *et al.* 1998; DeGabriel *et al.* 2010). Together, the above-named issues may constitute grounds for the listing of the South Gippsland koala population under the FFG Act.

South Gippsland research findings and management implications

1. Habitat extent and connectivity in South Gippsland

Fine scale population structure was detected in South Gippsland (chapter 8) which may be the result of past and present habitat, and population, fragmentation, or due to a preference of individual koalas to remain in their natal area. The genetic diversity of a population can be maintained by maximising population size and increasing gene flow between population clusters. Increasing habitat connectivity in the South Gippsland region would be a principal strategy for conserving the genetic diversity of koalas in the region and may also assist future movements of animals and populations in response to climate change. Coupling the genetic data generated in this study with landscape data would aid in identification of landscape factors that may impede or facilitate koala movements (Storfer *et al.* 2006). In Canada for example, landscape genetics has been used to show that rivers and roads were highly impeding movement of white footed mice, while dense fragments of forest were found to facilitate movement between large habitat patches by acting as stepping stones (Marrotte *et al.* 2014).

Climatic and landscape modelling may inform strategies to re-establish large scale connectivity (Nuñez *et al.* 2013). In their study of kinkajous, a mobile arboreal species found in South America, Keeley *et al.* (2017) showed that while low quality habitat was not preferred when moving within the home range, decreased habitat quality did not pose a barrier to dispersal movements. Given that koalas can be quite mobile and are known to move through landscapes without highly suitable habitat (Matthews *et al.* 2007; Menkhorst 2008), flexibility in tree species used and positioning of corridors may be possible (Keeley *et al.* 2017). Re-establishing habitat patches of sufficient size and within an appropriate distance between other habitat patches may therefore be one potential strategy for increasing gene flow on a large scale.

Outside of areas where well defined population clusters were detected, sampled koalas appeared to represent dispersers from the main population clusters (chapter 8). A lack of suitable habitat in the region could potentially be limiting successful recolonisation of areas outside of the main population clusters. Increasing the extent of koala habitat in South Gippsland would therefore be a key measure to maximise population size and hence retain genetic diversity.

Improving habitat quality in the region also has the potential to improve koala health. This study (chapter 9) identified high rates of infection with *C. pecorum* in South Gippsland, especially in areas containing high quality habitat (and the greatest koala densities for the region). Previous research has noted a positive trend between chlamydial infection, habitat quality and body condition (McAlpine *et al.* 2017). The higher prevalence of chlamydial infection in high quality habitat, but higher levels of health, was hypothesised to be due to an increased capacity of koalas to cope with chlamydial infection due to adequate nutrition. This suggests that koalas living in fragmented or suboptimal habitat may be more prone to the

negative effects of chlamydial infection. Improving habitat may therefore decrease potential impacts of chlamydial related disease.

2. *Transmission of pathogens*

Another management and conservation consideration involves the transfer of pathogens among populations. This research identified a relatively high prevalence of *C. pecorum* of up to 77% and a KoRV incidence of 28% in South Gippsland (Chapter 9). Numerous strains of *C. pecorum* were identified within spatially restricted areas despite gene flow between those areas. Data presented in chapter 9 (addendum) suggests that KoRV may be impacting koala health, with KoRV prevalence being higher in individuals affected by road trauma or illness compared to wild sampled individuals.

The impact of *C. pecorum* on koala health in the South Gippsland koala population has not been studied. Evidence from other studies suggest that different strains of *C. pecorum* have differing levels of pathogenicity (Mohamad *et al.* 2008; Mohamad *et al.* 2014). Given that the potential health impacts of *C. pecorum* strains and KoRV on South Gippsland koalas are unclear, it would be prudent to minimise the movement of pathogens between different populations and regions.

Wildlife shelters play a significant role in conservation through the rehabilitation and release of sick or injured animals, however, housing of koalas at wildlife shelters is a potentially important circumstance influencing pathogen spread. Pathogenic infections are sometimes asymptomatic where signs of infection are not always apparent. Aside from *C. pecorum* and KoRV, numerous other known or unknown pathogenic organisms may also be harboured by particular populations. One example is herpesvirus which, incidentally, is found to be highly associated highly with the presence of *C. pecorum* (Stalder *et al.* 2015). Animals from widely different locations may be brought into close contact at wildlife shelters, a situation that may

facilitate the spread of pathogens between shelter animals with subsequent dissemination of those pathogens throughout potentially naïve populations when individuals are released back to their population of origin. In addition, rehabilitated animals are often released at locations other than the site of origin (Guy & Banks 2012; Reid 2014). This may introduce new pathogens to the resident population or decrease the chance of survival of the released individual due to exposure to novel pathogens.

Ensuring that wildlife shelter operators and carers are aware of the risks of pathogen transmission, and of protocols for quarantine and release to minimise those risks, are therefore important to minimise inter-population transmission of pathogens. This is also an important consideration for proposed translocations of koalas, which may apply even to translocations across very short distances, as demonstrated for *C. pecorum* in the Strzelecki Ranges, where spatial areas dominated by a particular chlamydial strain could be separated by only a couple of kilometres.

3. Collection of more data for Victorian populations

In Victoria, population data and government spending is largely directed toward the small handful of koala populations at unsustainable densities (Menkhorst 2008). The problem of overpopulation is devastating for habitats, other species and the koalas themselves (Menkhorst 2008). Management and research into this problem is therefore vital for conservation, but this is currently occurring at the expense of monitoring and conservation of the remainder of Victorian koala populations. Koala populations deriving from translocated individuals exist at both high and low densities in different areas of Victoria and there are numerous other Victorian koala populations for which density/abundance data are not available (Menkhorst 2004). Some Victorian populations may be in decline and in need of conservation attention. The conservation of island-derived populations is important in

Victoria as these populations represent a unique subset of koala diversity found Australia wide (island and island derived populations are a less diverse subset of koala populations in South Gippsland). Although theory predicts that populations with greater genetic diversity will have an increased chance of surviving future environmental changes (Frankham 2005; Frankham *et al.* 2012), there is also the possibility that, due to chance, certain populations derived from island individuals may persist, while the South Gippsland koala population may not.

While this study confirmed that the South Gippsland koala population is a remnant population that is unique from, and more diverse than, island derived populations (chapter 8), there is limited data for other Victorian koala populations (Menkhorst 2004) which may result in difficulties defining appropriate conservation priorities. Apart from the South Gippsland population, it is often assumed that all Victorian koala populations are derived from island translocations, though this may not always be the case (Menkhorst 2008). Wide scale genetic surveys of koala populations across the state are needed to search for and identify any additional remnant populations. Other remnant populations are likely to possess a somewhat different subset of genetic diversity to that which has been preserved in South Gippsland, so identifying and conserving other remnant genetic diversity in Victoria is of importance.

Limitations and development of further applications

In this study, replicate microsatellite genotyping was used to obtain DNA profiles, where microsatellite marker products, amplified via PCR, were separated using capillary electrophoresis. A major limitation of capillary electrophoresis for genotyping, however, is that, due to differences in the rates of electrophoretic migration, results obtained by different laboratories or using different platforms are not directly comparable (De Barba *et al.* 2016).

Another potential constraint for some studies are the costs associated with replicate microsatellite genotyping for large sample numbers.

The use of high throughput sequencing (HTS) to obtain microsatellite genotypes from faecal samples has been demonstrated by De Barba *et al.* (2016). Utilising HTS for genotyping may address both limitations outlined above. Firstly, using HTS, microsatellite length is determined directly from DNA sequence data, so separate studies can be directly compared and, secondly, HTS is found to increase genotyping success compared to the capillary electrophoretic method and reduce costs by more than 40% (De Barba *et al.* 2016).

Developing methods for genotyping by HTS would be beneficial to the study of koala populations, as results could be easily standardised. This would allow different laboratories and interstate researchers to combine and compare data for the investigation of koala populations across their entire distribution, providing more in depth analyses of the species overall. Increased throughput, sensitivity and data transferability, along with reduced costs (De Barba *et al.* 2016) makes the development of microsatellite markers for genotyping using HTS a worthwhile priority in order to further improve non-invasive genotyping methods for koalas.

Data reliability was ensured in this study by screening DNA quantity and quality prior to genotyping as well as using strategies developed by Taberlet *et al.* (1996) and Valière *et al.* (2002) for replicate genotyping and Paetkau (2003) for identifying errors in final datasets. Greater sensitivity and cost reductions obtained using HTS for genotyping may, potentially, make it more time and cost effective to eliminate preliminary screening steps (Fig. 1) and genotype all scat samples appearing of good quality at the time of collection.

The suite of genetic methods that can employ non-invasively isolated koala DNA could also be widened by addition of markers such as single nucleotide polymorphisms (SNPs), which

have, in other species, been successfully applied to DNA isolated from faecal samples (Kraus *et al.* 2015; Fitak *et al.* 2016). SNPs are single base pair differences between sequences that are useful for population genetics (Brookes 1999; Coates *et al.* 2009). SNPs represent a high proportion of genomic variation and exist in both non-coding (neutral loci) and coding (genes) DNA (Brookes 1999). SNP variation within genes may allow the investigation of diversity under selection and variation involved in local adaptation, which would greatly extend the utility of DNA isolated from koala scats. Since SNPs are normally bi-allelic² (having only two alleles) while microsatellites are multi-allelic (having many alleles at a single locus), a greater number of SNP loci are required to achieve the same level of power as microsatellites (Morin *et al.* 2004). Due to the nature of SNPs (bi-allelic and smaller amplicon sizes), rates of genotyping error and amplification failure can be lower for SNPs than microsatellite markers when using non-invasively sourced DNA (Campbell & Narum 2009; Kraus *et al.* 2015). Since SNP data are also based on DNA sequence, this data is also directly transferable between studies and laboratories.

Further work to increase the number of analyses which may reliably utilise DNA isolated from koala scats would be ideal as this would increase the amount of information that can be gathered from DNA samples sourced from scats. For example, future development may involve the combination of microsatellite genotype, gender, mtDNA and both coding and non-coding SNP markers, as well as markers targeting *C. pecorum* and KoRV in one or more multiplex PCRs that could be sequenced together using HTS (Børsting & Morling 2015; De Barba *et al.* 2016).

Development of such a method would be of great benefit to koala research by providing a standardised tool that could be used to obtain data from koala populations Australia wide,

² Tri- and tetra- allelic loci are also possible, but extremely rare (Brookes, 1999).

including those that may be near impossible to sample otherwise (e.g. low density koala populations). As previously mentioned for HTS, the availability of such a tool would allow data to be directly compared and shared, thereby increasing the level of information that can be obtained across both fine and broad scales. Such a tool would not be limited by sample type and could utilise either non-invasively or invasively sourced DNA. Developing a standardised genetic tool for koalas would require input and involvement of koala researchers Australia wide to ensure that the requirements of all groups are incorporated.

In addition to using scats to obtain genetic information, scat samples also have the potential to provide extra information for particular studies. The measurement of faecal glucocorticoids to estimate physiological stress (Davies *et al.* 2013; Narayan *et al.* 2013), faecal cuticle analysis to identify dietary tree species (Ellis *et al.* 1999), analysis of faecal progesterone for the study of reproductive activity (Kusuda *et al.* 2009) and study of gut microbiomes using faecal samples (Alfano *et al.* 2015), are all examples of approaches which have been previously utilised in studies of koalas.

Conclusions

The research described in this thesis has demonstrated the reliability of non-invasive methods for obtaining genetic data pertaining to koalas and two of their pathogens, *C. pecorum* and KoRV. These methods will be of use to koala research by facilitating the rapid collection of data. The methods developed and presented in part one of the thesis were used to study the koala population in South Gippsland and have provided information relating to population structure, genetic diversity and the prevalence of *C. pecorum* and KoRV for koalas in the region. The South Gippsland koala population was shown to be unique from and more diverse than island derived populations, confirming its conservation significance. Together, these data

will help to inform any conservation or management actions in the region and also provide a baseline for future studies of genetic structure, diversity and pathogen prevalence.

Non-invasive genetic sampling can facilitate the collection of large amounts of data in a short period of time. As shown here, non-invasive genetic sampling has allowed a detailed survey of genetic diversity and pathogen carriage in South Gippsland. The methods developed for this study will also be of use for further data collection for koala populations with many potential opportunities for extending and refining the methods presented here. Even greater information is likely to be able to be obtained using non-invasive genetic sampling as technology increases and becomes more cost effective. Since koala populations are broadly distributed across Australia's east, developing standard methods that can be used by different research groups will greatly facilitate the ability to share and therefore analyse information at a broad scale therefore enabling koala conservation at the species level.

Habitats supporting native species have become increasingly fragmented and degraded since the colonisation of Australia by Europeans more than 200 years ago (Bradshaw 2012).

Increasing patch size and connectivity of koala habitats are key to the conservation of koala populations and the genetic diversity they encompass. Improving habitat across the koala's range is likely to increase population health and provide the greatest chance of surviving climate associated changes predicted for the 21st century, by maximising the retention of genetic diversity (and therefore adaptive potential) and facilitating animal movements to more suitable regions. Increasing the amount of continuous koala habitat across the koala's distribution will not only benefit koalas, but also the wide range of other forest dwelling species facing similar future challenges.

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Appendices





Appendix 1 | Data accessibility

Data collected during the course of this project can be accessed on Figshare (<https://figshare.com/>)

Sample database

<https://doi.org/10.6084/m9.figshare.5656120.v1>

The Microsoft Access file contains individual koalas sampled, replicate and consensus genotypes using 12 microsatellite markers, mitochondrial DNA (mtDNA) haplotypes for the mtDNA control region, the cytochrome b gene and DNA spanning genes for NADH dehydrogenase subunits 5 and 6, results of PCR assays for the detection of *Chlamydia pecorum* and koala retrovirus (KoRV-A) and genotypes for the *C. pecorum ompA* gene and KoRV-A *env* gene for some positive samples. A short version of the database is also provided in Microsoft Excel format.

DNA sequence data

<https://doi.org/10.6084/m9.figshare.5656159.v1>

FASTA files containing DNA sequence data for the koala mtDNA control region, koala mtDNA cytochrome b gene, koala mtDNA NADH dehydrogenase subunit 5 and 6 genes, *Chlamydia pecorum ompA* gene detected in koala scats and the KoRV-A *env* gene detected in koala DNA.

The above DNA sequence data are also available on Genbank under accession numbers **KY979201 - KY979210** (mtDNA control region), **KY979211 - KY979220** (mtDNA cytochrome b), **KY979221 - KY979230** (mtDNA NADH dehydrogenase subunit 5-6), **KY913821 - KY913837** (*Chlamydia pecorum ompA*) and **KY979231 - KY979233** (KoRV *env*)

GIS shapefile

<https://doi.org/10.6084/m9.figshare.5666629.v1>

Shapefile containing the main genetic results including results of population structure analyses, mtDNA haplotypes and *C. pecorum* and KoRV status.



Appendix 2 | Sample summaries

Table A1 Summary of total sample numbers obtained from each region.

Region	Contributed by	Number of individuals sampled
South Gippsland	Community/FOSK	87
	FW	81
	HVP/HF	33
	OEH	37
	SAWS	45
	S. Zent	35
	SAWS (biopsies)	36
Wilsons Promontory	Jim Whelan	10
Cape Otway	Deakin University	96
Raymond Island	FW	40
Koala Conservation Centre	FW	23
	MK	14
French Island	SAWS	9
Central Gippsland	SAWS	16
	SAWS (biopsies)	12
Victoria - other	Community/FOSK	5
	FW	3
	SAWS	7
	SAWS (biopsies)	2
South East NSW	OEH	24
North East NSW	PMKH	29
South East QLD	OWAD	13
	S. FitzGibbon and B. Ellis	4
Total		661

FOSK Friends of the Strzelecki Koala, **FW** Faye Wedrowicz, **HVP/HF** Hancock Victoria Plantations and Hazelwood Forestry, **OEH** Chris Allen (NSW Office of Environment and Heritage), **SAWS** Colleen Wood (Southern Ash Wildlife Shelter), **MK** Marwar Karsa, **PMKH** Port Macquarie Koala Hospital, **OWAD** Olivia Woosnam (OWAD Environment).

Table A2 Summary of genotypes obtained after removal of duplicates and poorly performing samples.

Region	Individuals sampled	Sent for genotyping	Samples with more than eight successfully amplified loci	Samples after the removal of duplicates
South Gippsland	364	291	273	221
Cape Otway	96	61	54	50
French Island	9	15	15	9
Raymond Island	41	39	33	31
Central Gippsland	28	29	28	19
Koala Conservation Centre	37	45	40	32
Victoria - other	16	14	10	9
South East New South Wales	24	19	15	12
North East New South Wales	29	27	24	24
South East Queensland	17	12	12	12
Total	661	552	504	419

Appendix 3 | Translocation data

Data within the following pages were obtained from Martin (1989)¹, Emmins (1996)² and information kindly provided by Peter Menkhorst from the Arthur Rylah Institute for Environmental Research. Translocation data are presented in Table A4 according to bioregion (shown in Figure A2).

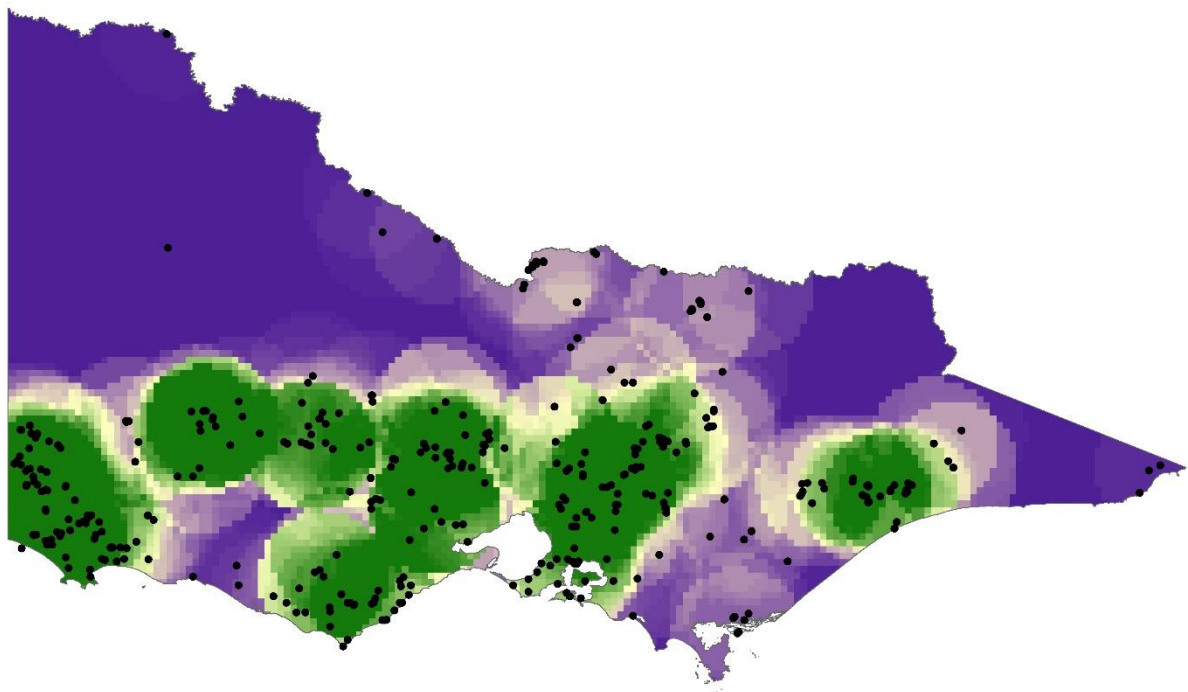


Figure A1 The distribution of koala release sites in Victoria. Black dots indicate release sites. Shading represents the density of koala release sites within different regions, ranging from low (purple), intermediate (cream) to high (green).

¹ Martin RW (1989) Draft management plan for the conservation of the koala (*Phascolarctos Cinereus*) in Victoria: a report to the Department of Conservation, Forests, and Lands, Victoria Department of Conservation, Forests, and Lands, Melbourne.

² Emmins JJ (1996) The Victorian koala: Genetic heterogeneity, immune responsiveness and epizootiology of Chlamydiosis, Monash University.

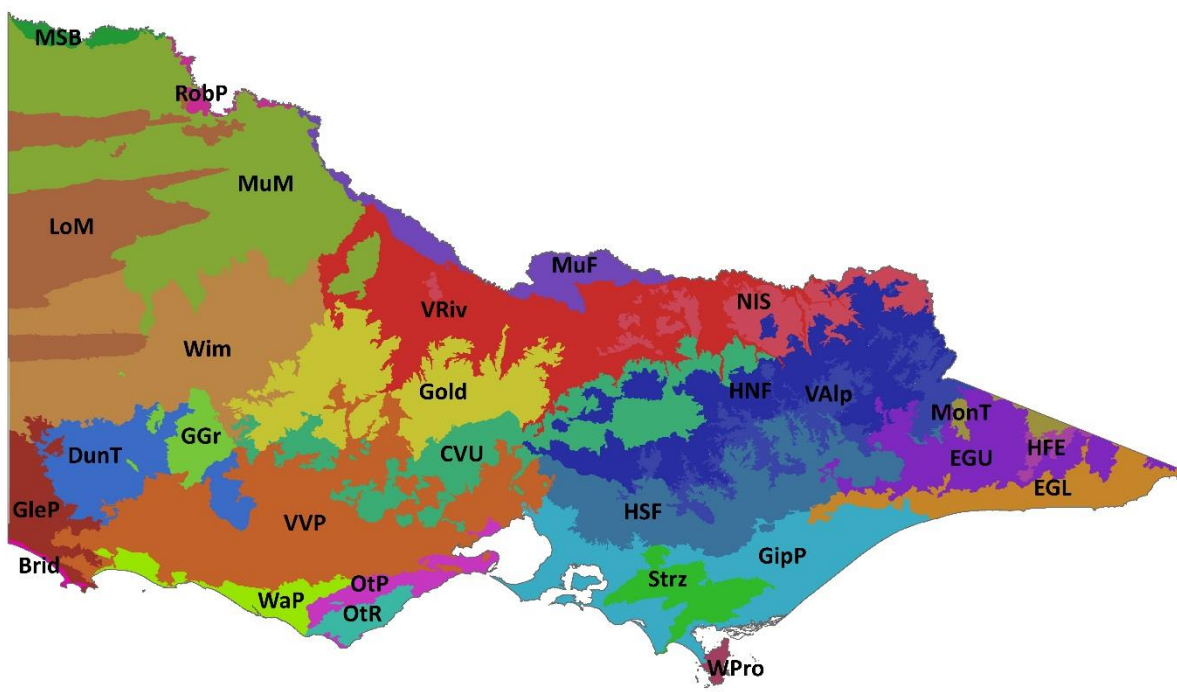


Figure A2 Map of Victoria showing the locations of the bioregions mentioned in the following pages. Bioregion data obtained from Department of Environment, Land, Water and Planning via the data.vic.gov.au website. The bioregion names for the codes used in the map are given below.

Table A3 List of bioregion codes and their corresponding names

<i>Code</i>	<i>Bioregion name</i>	<i>Code</i>	<i>Bioregion name</i>
Brid	Bridgewater	MuF	Murray Fans
CVU	Central Victorian Uplands	MuM	Murray Mallee
DunT	Dundas Tablelands	MSB	Murray Scroll Belt
EGL	East Gippsland Lowlands	NIS	Northern Inland Slopes
EGU	East Gippsland Uplands	OtP	Otway Plain
GipP	Gippsland Plain	OtR	Otway Ranges
GleP	Glenelg Plain	RobP	Robinvale Plain
Gold	Goldfields	Strz	Strzelecki Ranges
GGr	Greater Grampians	VAIp	Victorian Alps
HFE	Highlands – Far East	VRiv	Victorian Riverina
HNF	Highlands – Northern Fall	VVP	Victorian Volcanic Plain
HSF	Highlands – Southern Fall	WaP	Warrnambool Plain
LoM	Lowan Mallee	WPro	Wilsons Promontory
MonT	Monaro Tablelands	Wim	Wimmera

Table A4 Summary of koala translocations by bioregion**Central Victorian Uplands (CVU)**

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1927	FI	Camels Hump	1	-37.39	144.593	Mount Macedon	4999624
1931	FI	Camels Hump	6	-37.39	144.593	Mount Macedon	4999647
1942	PI	Creswick	23	-37.44	143.91	Cabbage Tree	4999508
1942	PI	Jubilee Lake Reserve	26	-37.357	144.16	Daylesford	4999509
1943	PI	Creswick	34	-37.44	143.91	Cabbage Tree	4999666
1944	PI	Brisbane Ranges	150	-37.824	144.226	Staughton Vale	4999510
1944	QI	Brisbane Ranges	155	-37.824	144.226	Staughton Vale	4999608
1944	QI	Creswick	33	-37.44	143.91	Cabbage Tree	4999613
1944	QI	Jubilee Lake Reserve	36	-37.357	144.16	Daylesford	4999614
1944	PI	Kyneton	38	-37.257	144.46	Kyneton	4999622
1944	CI	Enders Hill		-37.39	144.326	Trentham	4999637
1944	QI	Kyneton	38	-37.257	144.46	Kyneton	4999657
1944	QI	Cranneys Hill	63	-37.39	144.343	Trentham	4999658
1944	PI	Jubilee Lake Reserve	32	-37.357	144.16	Daylesford	4999683
1945	PI	Seymour	6	-37.04	145.143	Seymour	4999633
1945	PI	Brisbane Ranges	108	-37.824	144.226	Staughton Vale	4999668
1951	PI	Cranneys Hill	32	-37.39	144.343	Trentham	4999674
1952	PI	Bacchus Marsh-Macedon	46	-37.507	144.51	Bullengarook	4999504
1952	PI	Creswick	23	-37.44	143.91	Cabbage Tree	4999690
1953	PI	Mt Cole State Forest	83	-37.34	143.276	Raglan	4999525
1953	PI	Creswick	12	-37.44	143.91	Cabbage Tree	4999691
1954	FI	Mt Cole State Forest	161	-37.34	143.276	Raglan	4999626
1956	FI	Creswick	12	-37.44	143.91	Cabbage Tree	4999552
1957	PI	Hanging Rock Reserve	23	-37.34	144.61	Hesket	4999534
1957	FI	Elmhurst	100	-37.19	143.26	Elmhurst	4999554
1957	FI	Mt Cole State Forest	265	-37.34	143.276	Raglan	4999580
1957	FI	Brisbane Ranges	171	-37.824	144.226	Staughton Vale	4999653
1957	WI	State Forest	38	-37.34	143.276	Raglan	4999693
1957	PI	Brisbane Ranges	92	-37.824	144.226	Staughton Vale	4999698
1965	FI	Flinders Peak	11	-37.94	144.443	Little River	4999601
1967	?	Coller Bay	25	-37.19	145.843	Devils River	4999616
1973	PI	Coller Bay, Fraser National Park	36	-37.19	145.843	Devils River	4999512
1976	PI	Coller Bay, Fraser National Park	?	-37.19	145.843	Devils River	4999702
1977	FI	Brisbane Ranges	49	-37.824	144.226	Staughton Vale	4999703
1977	FI	Flinders Peak	32	-37.94	144.443	Little River	4999704
1987	FI	Jubilee Lake Reserve	16	-37.357	144.16	Daylesford	4999553

Central Victorian Uplands (CVU)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1987	FI	Coller Bay, Fraser National Park	?	-37.19	145.843	Devils River	4999559
1987	FI	Linton Flora And Fauna Reserve	12	-37.69	143.576	Linton	4999572
1987	FI	The Camels Hump	41	-37.39	144.593	Mount Macedon	4999576
1987	FI	Enders Hill	23	-37.39	144.326	Trentham	4999594
1987	FI	Enfield	25	-37.757	143.81	Enfield	4999716
1988	SP	Coller Bay	46	-37.19	145.843	Devils River	4999717
1988	SP	Cobaw State Forest	19	-37.29	144.643	Cobaw	4999719
1988	SP	Clear Water Creek	28	-37.44	144.343	North Blackwood	4999720
1989	SP	Pryrnees State Forest	22	-37.134	143.344	Glenpatrick	6185
1989	SP	Mt Beckworth State Forest	23	-37.313	143.725	Mount Beckworth	6186
1989	FI	Haddon Common	30	-37.588	143.737	Bunkers Hill	6202
1989	?	Pyrenees State Forest	18	-37.134	143.349	Glenpatrick	4999010-4999027
1989	?	Mt Beckworth State Forest	21	-37.315	143.727	Mount Beckworth	4999028-4999048
1989	?	Haddon Common	23	-37.585	143.737	Bunkers Hill	4999902-4999785
1990	SP	Dereel	37	-37.824	143.74	Dereel	6215
1990	SP	Creswick Koala Park	4	-37.439	143.913	Cabbage Tree	6218
1990	SP	Mt Cole	60	-37.329	143.248	Buangor	6216-6217
1991	SP	Mt Macedon	30	-37.329	144.598	Newham	106336
1991	SP	Lenderderg State Park	25	-37.495	144.361	Lerderderg	106338
1991	SP	Invermay	7	-37.502	143.891	Invermay	106346
1991	FI	Trentham State Forest	38	-37.351	144.231	Wheatsheaf	106332-106334
1992	FI	Cobaw State Forest	36	-37.249	144.619	Cobaw	6259
1992	FI	Daylesford State Forest	37	-37.511	144.328	Dales Creek	6254-6258
1993	FI	Hepburn Regional Park	19	-37.389	144.119	Sailors Falls	22610-22615
1993	FI	Enfield	25	-37.769	143.739	Enfield	22622-22626
1993	SP	Mt Cole	29	-37.254	143.281	Glenlogie	22627-22642
1994	FI	Pyrenees State Park	32	-37.134	143.344	Glenpatrick	22646
1995	SP	Wombat State Forest	22	-37.399	144.319	Trentham	22671
1995	FI	Wombat State Forest	33	-37.404	144.322	Trentham	22651-22665
1996	FI	Coller Bay, Fraser National Park	28	-37.177	145.864	Devils River	22689-22713
1997	FI	Lal Lal State Forest	10	-37.697	144.047	Mount Doran	37462
1997	FI	Creswick State Forest	10	-37.445	143.924	Cabbage Tree	37463
1997	FI	Enfield Forest Park	10	-37.746	143.777	Enfield	37464

Central Victorian Uplands (CVU)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1997	FI	Lerderderg State Park	44	-37.507	144.43	Lerderderg	37467-37469
1997	SP	Saltwater Creek, Wombat State Forest	16	-37.473	144.44	Lerderderg	37473-37475
1997	CR/B	Cobaw State Forest	16	-37.239	144.642	Cobaw	37476-37477
1998	FR	Ben Major	102	-37.363	143.447	Waterloo	60990-60993
1998	FR	Wombat Forest	193	-37.431	144.233	Bullarto South	61010-61013
2001	ME	Ben Major Flora Reserve	35	-37.318	143.391	Chute	70328
2001	ME	Black Range Scenic Reserve	27	-37.117	142.756	Black Range	70359
2001	ME	Buangor State Park	52	-37.312	143.206	Bayindeen	70329-70330
2001	ME	Langi Ghiran State Park	79	-37.311	143.075	Dobie	70356-70358
2002	ME	Langi Ghiran State Park	42	-37.324	143.099	Warrak	80191-80219
2002	ME	Mt Buangor State Park	43	-37.326	143.243	Buangor	80268-80300

Dundas Tablelands (DunT)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1996	TH	Kanagulk Streamside Reserve	22	-37.151	141.886	Kanagulk	23064
1996	TH	Kanagulk Streamside Reserve	21	-37.151	141.886	Kanagulk	23070
1997	TH	Digby/Merino	20	-37.755	141.473	Killara	66553
1998	FR	Fulhams	49	-37.158	141.869	Kanagulk	60999
1998	FR	Glendinning	228	-37.311	141.961	Vasey	61000
1999	?	Bahgallah Bushland Reserve	13	-37.646	141.404	Sandford	60011
1999	?	Bahgallah Bushland Reserve	24	-37.654	141.402	Sandford	60019
2001	ME	Fulham Streamside Reserve	34	-37.15	141.863	Kanagulk	70351
2002	ME	Fulham Streamside Reserve	34	-37.149	141.874	Kanagulk	79886-79912
2002	ME	Nangeela	148	-37.541	141.259	Corndale	80301-80401

East Gippsland Lowlands (EGL)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1982	FI	Totem Point	51	-37.49	149.776	Mallacoota	4999577
1986	FI	Nicolson River	52	-37.707	147.826	Wiseleigh	4999539
1988	FI	Monkey Creek	40	-37.64	147.876	Double Bridges	4999724
1988	FI	Gorman Point	26	-37.64	147.543	Bullumwaal	4999725
1989	?	Mt Little Dick	1	-37.69	147.843	Bruthen	100536
1991	?	Genoa-Mallacoota Road	2	-37.699	149.62	Wingan River	104814
1993	?	Mallacoota-Genoa Road	3	-37.529	149.69	Gipsy Point	112007, 112121
1996	SP	Bullumwaal	14	-37.642	147.535	Bullumwaal	22716
1996	SP	Ramrod Creek, Bruthen	14	-37.678	147.833	Bruthen	22717
1996	CR/B	Melwood	14	-37.768	147.549	Ellaswood	22718- 22719
1997	SI	Musk Gully	33	-37.717	147.498	Melwood	32983
1997	SI	Beynons Road, Melwood	40	-37.774	147.541	Ellaswood	32984
1997	SI	Bullumwaal	46	-37.642	147.535	Bullumwaal	32987
1997	SI	Boggy Creek	33	-37.651	147.544	Bullumwaal	32995
1997	SI	Nicholson River	30	-37.676	147.713	Waterholes	32997
1997	SI	Ramrod Creek, Bruthen	36	-37.679	147.833	Bruthen	32998
1997	SI	Musk Gully	33	-37.717	147.498	Melwood	37434
1997	SI	Boggy Creek, Bullumwall	33	-37.651	147.544	Bullumwaal	37448
1997	SI	Nicholson River	30	-37.676	147.713	Waterholes	37450
1997	SI	Mia Mia Track, Melwood	32	-37.732	147.53	Melwood	32985- 32986
1997	SI	Melwood	72	-37.774	147.541	Ellaswood	37435- 37436
1997	SI	Bullumwall	46	-37.642	147.535	Bullumwaal	37437- 37438
1997	SI	Mt Taylor	50	-37.727	147.635	Clifton Creek	37444- 37445
1997	SI	Ramrod Creek, Bruthen	35	-37.679	147.833	Bruthen	37451- 37452
1997	SI	Pea Hill Road, Melwood	42	-37.722	147.508	Melwood	37453- 37454
1944	QI	Hospital Creek, Buchan Reservoir	36	-37.507	148.193	Buchan	4999610
1957	PI	Hospital Creek, Buchan Reservoir	121	-37.507	148.193	Buchan	4999505
1957	PI	Hospital Creek	89	-37.323	148.043	Wulgulmerang West	4999628
1960	FI	Buchan Reservoir	53	-37.507	148.193	Buchan	4999542
1960	FI	Hospital Creek	135	-37.323	148.043	Wulgulmerang West	4999584
1988	FI	Nicholson River	21	-37.64	147.743	Fairy Dell	4999726
1991	SP	Gelantipy	27	-37.222	148.255	Gelantipy	106337

East Gippsland Lowlands (EGL)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1991	SP	Buchan	22	-37.458	148.154	Buchan	106341-106342
1996	FI	Monkey Creek, Bruthen	28	-37.625	147.847	Double Bridges	22688
1997	SI	Clifton Creek	125	-37.689	147.712	Fairy Dell	32988-32993
1997	SI	Clifton Creek	70	-37.689	147.712	Fairy Dell	37439-37443

Greater Grampians (GGr)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1947	CR	The Grampians	12	-37.074	142.46	Halls Gap	4999618
1947	CR	Grampians	12	-37.074	142.476	Halls Gap	4999688
1948	PI	Grampians	16	-37.074	142.476	Halls Gap	4999673
1957	FI	Grampians	611	-37.14	142.526	Halls Gap	4999563
1981	FI	Grampians	30	-37.574	142.376	Dunkeld	4999564
1999	?	Lake Bellfield, Grampians National Park	109	-37.193	142.55	Halls Gap	60025-60026, 60030
1999	?	Lake Wartook, Grampians National Park	104	-37.076	142.457	Halls Gap	60029, 60036
2000	?	Grampians National Park	315	-37.227	142.436	Bellfield	60051-60052, 60056-60065
2000	?	Grampians National Park	44	-37.334	142.662	Moyston	60071-60073
2001	ME	Grampians National Park	36	-37.122	142.531	Halls Gap	70346
2001	ME	Moora, Grampians National Park	119	-37.173	142.429	Glenisla	70305-70308, 70310-70313
2001	ME	Mt Dundas Scenic Reserve	66	-37.461	141.935	Gatum	70334-70335
2001	ME	Grampians National Park	124	-37.078	142.365	Zumsteins	70347-70348, 70350, 70353
2002	ME	Grampians National Park	184	-37.511	142.426	Bornes Hill	79913-80048

Gippsland Plain (GipP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1923	FI	Phillip Island	50	-38.49	145.26	Cowes	4999642
1930	FI	Mornington Peninsula	2	-38.407	144.826	St Andrews Beach	4999625
1930	FI	Mornington Racecourse	?	-38.24	145.043	Mornington	4999644
1930	FI	Warneet East	45	-38.24	145.293	Warneet	4999645
1930	FI	Chinaman Island	15	-38.244	145.313	Warneet	4999646
1931	FI	Warneet East	30	-38.24	145.293	Warneet	4999676
1931	FI	Chinaman Island	30	-38.244	145.313	Warneet	4999677
1932	FI	Warneet East	60	-38.24	145.293	Warneet	4999678
1933	FI	Warneet East	30	-38.24	145.293	Warneet	4999679
1944	PI	Gurdies	19	-38.373	145.593	Woodleigh	4999515
1944	PI	Yallock Outfall Floodway	20	-38.207	145.543	Monomeith	4999535
1944	QI	Junction Village	34	-38.123	145.293	Junction Village	4999612
1944	QI	Baxter	65	-38.207	145.16	Baxter	4999659
1944	QI	Cranbourne Road Reserve	308	-38.207	145.243	Pearcedale	4999662
1944	QI	Mornington Racecourse	84	-38.24	145.043	Mornington	4999663
1944	?	Quail Island	?	-38.227	145.281	Cannons Creek	4999694
1945	PI	Hedley	38	-38.657	146.51	Hedley	4999619
1945	PI	Eye Swamp	69	-38.773	146.543	Snake Island	4999634
1945	FI	Hedley	32	-38.657	146.51	Hedley	4999652
1945	FI	Eye Swamp	64	-38.773	146.543	Snake Island	4999687
1947	PI	Warneet East	32	-38.24	145.293	Warneet	4999672
1952	CI	Goat Island	4	-37.79	145.176	Donvale	4999617
1952	PI	Chinaman Island	5	-38.244	145.313	Warneet	4999675
1952	CR	Chinaman Island	6	-38.244	145.313	Warneet	4999689
1953	PI	Raymond Island	32	-37.923	147.76	Raymond Island	4999528
1957	FI	Chinaman Island	48	-38.244	145.313	Warneet	4999547
1965	FI	Chinaman Island	?	-38.244	145.313	Warneet	4999598
1966	MA	Waddy Island	12	-37.973	147.743	Ocean Grange	4999635
1972	FI	Sandy Point, Western Port	20	-38.396	145.167	Somers	4999589, 4999695
1977	PI	Cannon Creek	28	-38.223	145.326	Warneet	4999506
1977	PI	Point Norman	28	-38.64	145.743	Inverloch	4999519
1977	PI	Chinaman Island	30	-38.244	145.313	Warneet	4999533
1980	FI	Cannon Creek	17	-38.223	145.326	Warneet	4999543
1981	FI	Waddy Island	12	-37.973	147.743	Ocean Grange	4999570
1981	FI	Yarra Derran Reserve	5	-37.823	145.193	Mitcham	4999585
1982	FI	Waddy Island	39	-37.973	147.743	Ocean Grange	4999709
1985	FI	Lysterfield Hill	6	-37.957	145.276	Lysterfield South	4999548

Gippsland Plain (GipP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1985	SP	Mornington Peninsula	10	-38.457	144.943	Flinders	4999606
1986	?	Chinaman Island	6	-38.244	145.313	Warneet	4528808
1986	FI	Holey Hill	24	-38.223	146.926	Willung	4999567
1986	SP	Clumps Gutter	12	-38.673	146.593	Hedley	4999604
1986	SP	Devils Bend Reservoir	10	-38.257	145.11	Moorooduc	4999605
1986	SP	The Bryars	10	-38.307	145.01	Safety Beach	4999715
1987	?	Chinaman Island	3	-38.244	145.313	Warneet	4528809
1987	?	McLeod Point	6	-38.507	145.343	Newhaven	4528811-4528812
1991	SP	Arthurs Seat State Park	14	-38.359	144.943	Mccrae	106339-106340
1997	?	Phillip Island	10	-38.465	145.235	Cowes	119794
1997	?	French Island	10	-38.373	145.376	French Island	119795
1990s	SI	Gellions Run	45	-38.623	146.626	Gelliondale	122587

Glenelg Plain (GleP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1970	FI	Lower Glenelg National Park	44	-38.074	141.276	Drik Drik	4999561
1970	FI	Mt Richmond	44	-38.274	141.426	Gorae West	4999582
1982	FI	Portland Creek	49	-37.924	141.66	Condah	4999551
1985	FI	Lower Glenelg National Park	57	-38.074	141.276	Drik Drik	4999712
1989	FI	Crawford River Regional Park	16	-37.935	141.506	Hotspur	6200
1989	?	Crawford River Regional Park	12	-37.931	141.508	Hotspur	4999861-4999873
1993	TH	Mt Clay	23	-38.205	141.68	Narrawong	23051-23052
1995	TH	Lower Glenelg National Park	21	-38.019	141.179	Drik Drik	23056
1995	TH	Parrican Bend, Lower Glenelg National Park	24	-38.066	141.251	Drik Drik	23057
1995	TH	Lower Glenelg National Park	53	-38.025	141.16	Nelson	23060-23061
1996	TH	Strathdownie	23	-37.688	141.22	Bahgallah	23065
1996	TH	Mt Clay	15	-38.205	141.68	Narrawong	23066
1996	TH	Cemetary Swamp	21	-37.576	141.155	Lindsay	23067
1996	TH	Casterton	21	-37.576	141.155	Lindsay	23071
1996	FR	Hotspur	59	-37.873	141.569	Digby	23072-23074
1997	WR	Beniagha Swamp	6	-38.125	141.066	Nelson	60006

Glenelg Plain (GleP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1997	?	Lower Glenelg National Park	103	-38.021	141.154	Nelson	49998-49999, 60000-60002, 6005
1997	SP	Baileys Rock, Dergholm	35	-37.28	141.172	Dergholm	60003-60004
1997	TH	Hotspur	28	-37.886	141.582	Hotspur	66551-66552
1998	FR	Blackjack Track, Casterton	546	-37.612	141.161	Lindsay	60994-60998
1999	?	Hotspur Bushland Reserve	23	-37.932	141.558	Hotspur	60009
1999	?	Lower Glenelg National Park	15	-38.073	141.289	Drik Drik	60013
1999	?	Wirey Swamp Bushland Reserve	18	-37.637	141.104	Lindsay	60015
1999	?	Wirey Swamp Bushland Reserve	20	-37.637	141.104	Lindsay	60017
1999	?	Tooloy Flora Reserve	46	-37.533	141.098	Lake Mundi	60024
1999	?	Beniagh Wildlife Reserve	17	-37.217	141.065	Poolaijelo	60027
1999	?	Mageppa Bushland Reserve	21	-37.184	141.128	Poolaijelo	60028
1999	?	Crawford River Regional Park	57	-37.902	141.425	Winnap	60008, 60010
1999	?	Sharams Road, Dergholm State Park	75	-37.246	141.14	Poolaijelo	60012, 60014, 60018, 60020-60021
1999	?	Youpayang Block, Dergholm State Park	96	-37.335	141.344	Chetwynd	60022-60023
1999	?	Lake Mundi Wildlife Reserve	85	-37.484	141.061	Lake Mundi	60031-60032
1999	?	Glenelg River Streamline Reserve	39	-37.839	141.252	Dartmoor	60034, 60037
1999	?	Drajurk State Forest	24	-37.515	141.132	Lake Mundi	60047-60049
2000	?	Dergholm State Park	78	-37.248	141.187	Powers Creek	60066-60069
2000	?	Lake Mundi, Casterton	60	-37.458	141.032	Lake Mundi	60070, 60074
2000	?	Lower Glenelg National Park	19	-38.097	141.295	Mount Richmond	70336
2001	ME	Tooloy Flora Reserve	32	-37.519	141.114	Lake Mundi	70337
2001	ME	Lower Glenelg National Park	17	-38.097	141.26	Drik Drik	70345
2001	ME	Tooloy Flora Reserve	27	-37.68	141.262	Bahgallah	70349
2001	ME	Lower Glenelg National Park	10	-38.098	141.301	Mount Richmond	70352
2001	ME	Lake Mundi Wildlife Reserve	22	-37.477	141.011	Lake Mundi	70354
2001	ME	Roseneath Flora Reserve	37	-37.359	141.124	Dergholm	

Glenelg Plain (GleP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
2001	ME	Glenelg River Streamside Reserve	47	-37.825	141.249	Dartmoor	70326-70327
2001	ME	Bahgallah Streamside Reserve	79	-37.676	141.254	Bahgallah	70331-70333
2001	ME	Youpayang Block, Dergholm	141	-37.306	141.276	Dergholm	70338-70342
2001	ME	Wirey Swamp Bushland Reserve	60	-37.64	141.107	Lindsay	70343-70344
2002	ME	Brimboal State Forest	75	-37.357	141.364	Chetwynd	79806-79860
2002	ME	Drajurk State Forest	34	-37.638	141.187	Lindsay	79861-79885
2002	ME	Lake Mundi Wildlife Reserve	40	-37.421	141.052	Lake Mundi	80165-80190
2002	ME	Mageppa Bushland Reserve	23	-37.186	141.132	Poolaijelo	80255-80267
2002	ME	Crawford River Regional Park	38	-37.917	141.54	Hotspur	80402-80423
2002	ME	Roseneath State Forest	120	-37.52	141.215	Corndale	80424-80515
2002	ME	Wilkin Flora And Fauna Reserve	55	-37.683	141.263	Bahgallah	80516-80539
2002	ME	Winayung State Forest	70	-37.871	141.6	Grassdale	80540-80581
2002	ME	Wirey Swamp Bushland Reserve	21	-37.648	141.103	Lindsay	80582-80582

Goldfields (Gold)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1941	PI	Mt Alexander Koala Reserve	54	-37.007	144.31	Harcourt North	4999664, 4999681
1942	PI	Beehive Gully	25	-37.324	144.143	Hepburn Springs	4999517
1943	PI	Wesley Hill	38	-37.074	144.226	Castlemaine	4999665
1944	PI	Avoca	18	-37.09	143.493	Avoca	4999502
1944	PI	Metcalf	17	-37.107	144.443	Metcalf	4999523
1944	QI	Wesley Hill	105	-37.074	144.226	Castlemaine	4999661
1944	PI	Mt Alexander Koala Reserve	152	-37.007	144.31	Harcourt North	4999685
1945	PI	Wesley Hill	73	-37.074	144.226	Castlemaine	4999667
1965	WI	Teddington Reservoir	30	-36.807	143.293	Stuart Mill	4999636
1982	FI	Beehive Gully	68	-37.324	144.143	Hepburn Springs	4999566
1987	FI	Glen Patrick Mountain Hut	41	-37.174	143.393	Amphitheatre	4999588
1989	SP	Bet Bet Creek, Timor State Forest	25	-36.954	143.742	Timor	6184
1989	?	Bet Bet Creek, Timor State Forest	21	-36.954	143.748	Timor	4999989-4999009

Goldfields (Gold)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1992	FI	Pyrenees State Forest	25	-37.011	143.212	Frenchmans	6260
1993	FI	Kara Kara State Park	24	-36.859	143.257	Redbank	22616
1996	FI	Pyrenees State Forest	35	-37.085	143.368	Percydale	22714-22715
1997	SP	Timor and Havelock Forest	23	-37.002	143.751	Simson	37472
2001	ME	Deep Lead Flora & Fauna Reserve	63	-37.001	142.728	Deep Lead	70315-70321
2001	ME	Ararat Hills Regional Park	9	-37.246	142.887	Norval	70322-70325

Highlands – Northern Fall (HNF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1940	FI	Jock Lookout	16	-37.523	145.76	Marysville	4999578
1941	PI	Strathbogie	30	-36.857	145.743	Strathbogie	4999530
1941	PI	Ruffy	30	-36.99	145.51	Ruffy	4999632
1944	QI	Strathbogie	36	-36.857	145.743	Strathbogie	4999609
1944	QI	Ruffy	32	-36.99	145.51	Ruffy	4999660
1944	PI	Strathbogie	32	-36.857	145.743	Strathbogie	4999682
1944	PI	Ruffy	32	-36.99	145.51	Ruffy	4999684
1944	PI	Strathbogie	39	-36.857	145.743	Strathbogie	4999686
1945	PI	Ruffy	35	-36.99	145.51	Ruffy	4999670
1945	PI	Strathbogie	75	-36.857	145.743	Strathbogie	4999671
1967	?	Mt Wombat	?	-36.857	145.676	Kelvin View	4999627
1985	FI	Jock Lookout	19	-37.523	145.76	Marysville	4999713
1987	FI	Mt Sugarloaf	45	-37.407	145.76	Buxton	4999545
1988	?	Blue Gum Flat	?	-37.287	145.958	Eildon	1663689
1988	SP	Mt Sugarloaf	42	-37.407	145.76	Buxton	4999718
1988	SP	Taylor Creek	15	-37.307	146.01	Eildon	4999723
1989	?	Kinglake National Park	29	-37.39	145.376	Glenburn	4999873-4999901
1989	?	Sappers Track, Eildon State Park	22	-37.325	146.15	Kevington	4999948-4999969
1989	?	Davons Flat, Eildon State Park	19	-37.191	146.354	Howqua Hills	4999970-4999988
1989	SP	Eildon State Park	56	-37.325	146.15	Kevington	6182-6183
1991	FI	Eildon State Park	40	-37.31	145.951	Eildon	106331
1991	FI	Marysville State Park	37	-37.502	145.803	Marysville	106335
1991	SP	Howqua Hills Historic Area	30	-37.2	146.317	Howqua Hills	106345
1994	FI	Eildon State Park	38	-37.19	146.351	Howqua Hills	22644
1994	FI	Eildon State Park	41	-37.325	146.15	Kevington	22645

Highlands – Northern Fall (HNF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1994	SP	Eildon	16	-37.31	145.951	Eildon	22647
1994	SP	Eildon State Park	30	-37.276	145.944	Eildon	22648
1995	FI	Cathedral State Park	34	-37.358	145.754	Taggerty	22649-22650
1995	FI	Cathedral State Park	25	-37.362	145.779	Taggerty	22668-22669
1996	CR/B	Kinglake National Park	13	-37.479	145.348	Kinglake Central	22672-22673
1996	FI	Eildon State Park	31	-37.312	145.953	Eildon	22674-22686
1997	FI	Howqua Hills Historic Area	42	-37.191	146.331	Howqua Hills	37465
1997	FI	Marysville State Forest	35	-37.502	145.766	Marysville	37466
1997	FI	Eildon National Park	38	-37.288	145.974	Eildon	37470
1997	FI	Marysville State Forest	38	-37.511	145.732	Marysville	37471
1998	?	Turramurra	1	-36.94	146.213	Tolmie	35189
1998	SP	Eildon National Park	17	-37.31	146.144	Jamieson	37483
1998	FI	Davons Flat, Eildon National Park	81	-37.194	146.337	Howqua Hills	37478, 37481
1998	CR/B	Eildon National Park	27	-37.325	146.15	Kevington	37484-37486
1999	?	Marysville State Forest	35	-37.559	145.681	Narbethong	60075
1999	?	Eildon National Park	34	-37.288	145.963	Eildon	60076
1999	?	Timbertop School	28	-37.126	146.304	Merrijig	60077
1999	?	Marysville State Forest	42	-37.564	145.671	Narbethong	60078
1999	?	Rubicon Dry Creek	33	-37.348	145.973	Eildon	60079
1999	?	Mansfield State Forest	27	-37.062	146.361	Sawmill Settlement	60080
1999	?	Marysville State Forest	13	-37.078	146.361	Sawmill Settlement	60081
1999	?	Big River Valley Lower	16	-37.39	146.074	Woods Point	60082
1999	?	Lower Rubicon	29	-37.31	145.818	Thornton	60083-60085
2004	FI	Mt Disappointment State Forest	415	-37.309	145.151	Strath Creek	99966-99975

Highlands – Southern Fall (HSF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1935	FI	Healesville Fauna Park	28	-37.707	145.626	Millgrove	4999648
1938	FI	National Park	6	-37.89	145.31	Upper Ferntree Gully	4999649
1939	FI	National Park	6	-37.89	145.31	Upper Ferntree Gully	4999680
1940	FI	National Park	6	-37.89	145.31	Upper Ferntree Gully	4999557
1944	PI	Healesville Fauna Park	35	-37.707	145.626	Millgrove	4999507
1944	PI	Hoddles Creek	41	-37.84	145.61	Hoddles Creek	4999518

Highlands – Southern Fall (HSF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1944	PI	Yarra Junction	40	-37.79	145.626	Yarra Junction	4999536
1944	QI	Healesville High School	64	-37.657	145.543	Healesville	4999620
1944	QI	Corranderk	64	-37.657	145.51	Healesville	4999656
1952	CI	Mcmahons Creek Pipeline	29	-37.707	145.843	Mcmahons Creek	4999629
1956	FI	Healesville High School	29	-37.657	145.543	Healesville	4999565
1957	PI	Watts River Valley	100	-37.64	145.576	Healesville	4999522
1957	FI	Corranderk	46	-37.657	145.51	Healesville	4999654
1958	PI	Watts River Valley	164	-37.64	145.576	Healesville	4999692
1960	FI	Corranderk	80	-37.657	145.51	Healesville	4999699
1965	FI	Moondarra Reservoir	?	-38.09	146.393	Yallourn North	4999579
1968	?	Olinda State Forest	?	-37.844	145.388	Olinda	103627
1970	FI	Andrews Hill	33	-37.573	145.36	Kinglake	4999569
1971	PI	Healesville High School	8	-37.657	145.543	Healesville	4999516
1972	FI	Healesville Fauna Park	?	-37.707	145.626	Millgrove	4999550
1973	PI	Ferntree Gully National Park	35	-37.89	145.31	Upper Ferntree Gully	4999511
1973	PI	Pinchgut Creek	26	-37.557	145.36	Kinglake	4999520
1974	PI	Butterfield Reservoir	29	-37.89	145.426	Monbulk	4999524
1975	PI	Glen Evart	6	-37.79	145.61	Yarra Junction	4999521
1976	PI	Watts River Valley	?	-37.64	145.576	Healesville	4999701
1981	FI	Andrews Hill	39	-37.573	145.36	Kinglake	4999705
1982	?	Narre Warren North	1	-37.957	145.293	Lysterfield South	4528959
1982	FI	Boola Boola State Forest	46	-38.057	146.593	Toongabbie	4999707
1983	FI	Lysterfield Lakes National Park	36	-37.957	145.31	Lysterfield	4999573
1985	FI	Warrandyte State Park	30	-37.757	145.226	Warrandyte	4999599
1985	FI	National Park	10	-37.89	145.31	Upper Ferntree Gully	4999711
1987	SP	Briagolong	20	-37.623	147.076	Toolome	4999607
1988	SP	Pound Bend, Warrandyte State Park	33	-37.757	145.226	Warrandyte	4999640
1988	SP	Tabberabera-Bullumwaal	22	-37.59	147.426	Ryans	4999722
1989	?	Delvin Park	?	-37.532	145.235	Kinglake West	4999925-4999947
1989	FI	Kinglake National Park	65	-37.528	145.235	Kinglake West	6201, 6203-6204, 6220-6221
1990	?	Warrandyte State Park	100	-37.734	145.209	North Warrandyte	4223
1990	SP	Olinda State Forest	41	-37.847	145.383	Olinda	6210-6212
1991	SP	O'Tooles Flat, Donnelly's Creek	21	-37.749	146.442	Toombon	106343
1991	FI	Loch Valley	39	-37.853	145.997	Noojee	106328-106330

Highlands – Southern Fall (HSF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1996	FI	Toorourrong Reservoir, Wallaby Creek Catchment	36	-37.473	145.16	Whittlesea	22687
1997	SI	Cowarr Weir	18	-37.993	146.649	Cowwarr	32974
1997	SI	Blue Pool	29	-37.779	147.113	Briagolong	32977
1997	SI	Toggle Hill	20	-37.689	147.042	Woolenook	32978
1997	SI	New Place Track	32	-37.63	147.036	Toolome	32979
1997	SI	Davey Knob	44	-37.666	147.206	Moornapa	32980
1997	SI	Sandy Creek, Mitchell	34	-37.616	147.423	Merrijig	37449
1997	SI	Scubby Creek	22	-37.724	147.02	Woolenook	32975-32976
1997	SI	Sandy Creek, Mitchell River National Park	78	-37.616	147.423	Merrijig	32994, 32996
1997	SI	Cowarr Weir, Boola State Forest	29	-37.993	146.649	Cowwarr	37421-37422
1997	SI	Scrubby Creek, Freestone Forest Block	22	-37.713	147.029	Woolenook	37423-37423
1997	SI	Freestone Forest Block	80	-37.779	147.113	Briagolong	37425-37427, 37428, 37430, 37432-37433
1997	SI	Near Cobbannah	112	-37.624	147.189	Cobbannah	37446-37447
1997	SI	Sandy Creek, Mitchell River National Park	44	-37.616	147.423	Merrijig	39281-39282
1997	SI	Reedy Creek Track	80	-37.624	147.189	Cobbannah	60102
1999	?	Blue Pool	16	-37.774	145.978	Loch Valley	60086-60089
1999	?	Loch Extension	25	-37.82	145.996	Loch Valley	60090-60092
1999	?	Icy Creek-Noojee	19	-37.815	145.988	Loch Valley	60093-60095
1999	?	O'Tooles-Merringtons	66	-37.75	146.44	Toombon	60096-60101
1999	?	Moondarra State Park	35	-38.035	146.332	Moondarra	60103-60107
1999	?	Scrubby Creek Road	20	-37.721	145.89	Mcmahons Creek	60108-60110
1999	?	Lloyds Knob Track	29	-37.7	146.01	Toorong	73486-73489
2001	FI	Bunyip State Park	137	-37.984	145.604	Maryknoll	

Murray Fans (MuF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1976	PI	Pental Island	?	-35.407	143.71	Pental Island	4999630
1976	PI	Ulupna Bridge	?	-35.857	145.443	Ulupna	4999700

Murray Fans (MuF)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1977	FI	Ulupna Bridge	97	-35.873	145.46	Strathmerton	4999595
1978	PI	Lower Moira	5	-36.107	144.91	Kanyapella	4999621
1979	FI	Lower Moira	21	-36.107	144.91	Kanyapella	4999568
1981	FI	Pental Island	27	-35.407	143.71	Pental Island	4999587
1989	FI	Loch Garry, Goulburn River	33	-36.242	145.31	Bunbartha	6198
1989	?	Barmah Yards	?	-35.973	144.976	Barmah	4999696
1989	?	Barmah State Forest	30	-35.932	145.001	Barmah	4999049-4999078
1989	?	Gunbower Island State Forest	42	-35.755	144.242	Cohuna	4999079-4999122
1989	SP	Barmah State Forest	34	-35.957	144.976	Barmah	4999727-4999760
1989	?	Loch Garry, Goulburn River	22	-36.243	145.316	Bunbartha	4999786-4999807
1989	SP	Goose Swamp & Rat Castle, Barmah State Forest	33	-35.932	145.001	Barmah	6187-6193
1989	SP	Gunbower Island State Forest	45	-35.758	144.243	Cohuna	6194-6196
1990	FI	Kanyapella Wildlife Reserve	38	-36.138	144.901	Kanyapella	6228
1990	FI	Barmah State Forest	37	-35.95	145.004	Barmah	6229
1990	FI	Barmah State Forest	39	-35.936	145.057	Picola West	6232
1990	?	Top Lake	1	-35.929	145.056	Picola West	102877
1995	FI	Yarrawonga Regional Park	42	-36.009	145.978	Yarrawonga	22666-22667
1998	FI	Barmah State Park	39	-35.996	144.943	Barmah	37479
1998	FI	Barmah State Forest	42	-35.932	145.06	Picola West	37482

Murray Mallee (MuM)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1995	?	Hopetoun	1	-35.824	142.185	Rainbow	115369

Northern Inland Slopes (NIS)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1965	FI	Chiltern Police Station	107	-36.157	146.626	Chiltern	4999546
1972	FI	Ryans Lookout	54	-36.307	146.193	Mount Bruno	4999597
1982	FI	Ryans Lookout	64	-36.307	146.193	Mount Bruno	4999710
1990	SP	Warby Ranges State Park	34	-36.293	146.193	Killawarra	6205-6209
1998	FI	Warby Ranges State Park	33	-36.311	146.182	Mount Bruno	37480

Otway Plain (OtP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1981	FI	Cape Otway	49	-38.874	143.526	Hordern Vale	4999544
1982	FI	Boonah Plantation	51	-38.374	143.96	Bambra	4999538
1985	FI	Lovat	15	-38.54	143.56	Gellibrand	4999571
1990	FI	Gum Gully, Otway State Forest	37	-38.477	143.511	Barongarook West	6230
1990	FI	Carlisle State Park	39	-38.575	143.422	Carlisle River	6231
1993	FI	Angahook-Lorne State Park	39	-38.448	143.795	Murroon	22617-22618
1993	FI	Bambra Coal Mine	27	-38.345	143.986	Winchelsea South	22619-22621
1998	FR	Banool, Otways	22	-38.54	143.579	Gellibrand	61001
1998	FR	Gellibrand White Peg, Otways	36	-38.473	143.512	Barongarook West	61005
1958	PI	Otway Ranges	294	-38.674	143.826	Grey River	4999514
1973	PI	Moggs Creek	53	-38.407	144.043	Wensleydale	4999501
1977	FI	Moggs Creek	46	-38.407	144.043	Wensleydale	4999537
1977	FI	Otway Ranges	50	-38.674	143.826	Grey River	4999562
1982	FI	Blanket Bay Creek	33	-38.824	143.56	Cape Otway	4999586
1982	FI	Moggs Creek	40	-38.407	144.043	Wensleydale	4999706
1982	FI	Otway Ranges	12	-38.674	143.826	Grey River	4999708
1985	FI	Lorne	26	-38.54	143.976	Lorne	4999574
1987	SP	Moggs Creek	35	-38.407	144.043	Wensleydale	4999602
1987	SP	King Creek	59	-38.54	143.76	Barwon Downs	4999603
1988	SP	Carlisle State Park	23	-38.607	143.426	Wyelangta	4999721
1990	?	Lorne	1	-38.54	143.96	Lorne	102553
1990	?	Kennett River	4	-38.674	143.843	Grey River	102554
1992	SP	Lake Elizabeth	13	-38.549	143.746	Barramunga	6262
1992	?	Grey River Road	2	-38.67	143.857	Grey River	107749, 108474
1992	FI	Lorne-Angahook State Park	39	-38.6	143.918	Separation Creek	6252-6253
1993	SP	Callahans Creek, Otway State Forest	16	-38.507	143.772	Barwon Downs	22643
1994	TH	Lavers Hill	14	-38.724	143.426	Johanna	23055
1998	FR	Banool Lardners Track, Otways	64	-38.555	143.612	Gellibrand	61002
1998	FR	Forrest Elizabeth Track, Otways	43	-38.549	143.746	Barramunga	61003
1998	FR	Gellibrand Meehans Road, Otways	28	-38.56	143.607	Gellibrand	61004
1998	FR	Grassy Creek Lorne, Otways	28	-38.48	144.027	Big Hill	61006
1999	?	Angahook Lorne State Park	19	-38.415	143.968	Boonah	60016

Robinvale Plain (RobP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1957	FI	Loch Island	6	-34.19	142.176	Nichols Point	4999575
1957	FI	Loch Island	6	-34.19	142.176	Nichols Point	4999655
1963	WI	Loch Island	6	-34.19	142.176	Nichols Point	4999623

Strzelecki Ranges (Strz)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1944	PI	Poowong	82	-38.357	145.776	Poowong	4999527
1952	CI	Glen Chromie Park	6	-38.173	145.943	Warragul	4999639
1991	?	Warragul	1	-38.158	145.911	Warragul	104040

Victorian Riverina (VRiv)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1928	FI	Bontharambo	3	-36.357	146.31	Wangaratta	4999638
1979	FI	Cheshunt	59	-36.773	146.426	Whitfield	4999600
1982	FI	Euroa	4	-36.757	145.576	Euroa	4999556
1982	FI	Otway Ranges	27	-35.707	143.826	Fairley	4999558
1989	FI	Goulbourn River	28	-36.517	145.315	Toolamba	6197
1989	?	15 km north of Wangaratta	?	-36.257	146.26	Killawarra	4999697
1989	SP	Ovens River Valley	25	-36.24	146.26	Killawarra	4999761-4999785
1989	?	Cemetary Bend, Goulburn River	24	-36.514	145.321	Toolamba	4999808-4999831
1990	SP	Goulburn River	41	-36.587	145.264	Murchison North	6213-6214
1995	SP	Ovens River State Forest	32	-36.23	146.251	Killawarra	22670

Victorian Volcanic Plain (VVP)

Year	Origin	Release site	No. koalas	Lat	Long	Nearest Locality	Reference number/s
1928	FI	Romsey	4	-37.357	144.76	Romsey	4999631
1928	FI	Lethbridge	4	-37.974	144.143	Russells Bridge	4999643
1939	FI	Pomborneit	17	-38.307	143.31	Stonyford	4999650
1943	PI	Coimadai	25	-37.624	144.61	Toolern Vale	4999531
1944	PI	South Dreeite	150	-38.174	143.476	Dreeite South	4999529
1945	?	Bates Point	?	-38.074	144.476	Avalon	4999651

Victorian Volcanic Plain (VVP)

Year	Origin	Release site	No. Koalas	Lat	Long	Nearest Locality	Reference number/s
1945	PI	South Dreeite	115	-38.174	143.476	Dreeite South	4999669
1952	PI	Winnap-Nelson	32	-38.057	141.293	Drik Drik	4999513
1954	FI	Floating Islands Reserve	550	-38.34	143.376	Pirron Yallock	4999590
1957	FI	Brisbane Ranges National Park	230	-37.924	144.276	Anakie	4999541
1970	FI	Framlingham	37	-38.257	142.71	Framlingham	4999560
1973	PI	Mt. Eccles National Park	30	-38.074	141.943	Bessiebelle	4999526
1975	PI	Bats Ridge Faunal Reserve	24	-38.34	141.593	Portland	4999503
1981	FI	Mt Napier	42	-37.907	142.076	Mount Napier	4999581
1981	FI	Bryan Swamp, The Grampians	16	-37.574	142.26	Karabeal	4999596
1982	FI	Mt. Eccles National Park	46	-38.074	141.943	Bessiebelle	4999591
1985	FI	Floating Islands Reserve	7	-38.34	143.376	Pirron Yallock	4999714
1989	FI	Mt Napier State Park	36	-37.873	142.033	Mount Napier	6199
1989	?	Mt Napier State Park	29	-37.873	142.031	Mount Napier	4999832-4999860
1990	FI	You Yangs Regional Park	72	-37.945	144.387	Little River	6219, 6222
1991	SP	Inverleigh Flora Reserve	16	-38.061	144.035	Inverleigh	106344
1992	SP	Mt Bolton	9	-37.354	143.657	Waubra	6261
1992	SP	Mt Bolton	11	-37.354	143.657	Waubra	6263
1993	TH	Homerton Block	42	-38.114	141.755	Homerton	23049-23050
1995	TH	Cobboboonee State Forest	46	-38.193	141.404	Mount Richmond	23058-23059
1996	TH	Cobboboonee State Forest	15	-38.193	141.404	Mount Richmond	23062
1996	TH	Tyrendarra North	21	-38.119	141.763	Homerton	23063
1996	TH	Myamyn	20	-37.997	141.68	Myamyn	23068
1996	TH	Annya State Forest	20	-38.019	141.634	Milltown	23069
1998	FR	Stoney Rises, Otways	23	-38.292	143.346	Stonyford	61009
1999	?	Bolwarra West Bushland Reserve	20	-38.287	141.589	Bolwarra	60007
2000	?	Lower Glenelg National Park	177	-38.007	141.369	Greenwald	60038-60046, 60050
2001	ME	Mt Clay Flora Reserve	13	-38.214	141.705	Narrawong	70355
2001	ME	Lower Glenelg National Park	63	-37.999	141.337	Drik Drik	70304, 70309, 70314, 70360
2002	ME	Annya State Forest	151	-38.052	141.623	Milltown	79626-79731
2002	ME	Homerton State Forest	82	-38.119	141.746	Homerton	80049-80115

Victorian Volcanic Plain (VVP)

Year	Origin	Release site	No. Koalas	Lat	Long	Nearest Locality	Reference number/s
2002	ME	Hotspur State Forest	66	-37.986	141.438	Greenwald	80116-80164
2002	ME	Lower Glenelg National Park	42	-38.031	141.361	Greenwald	80220-80254

Warrnambool Plain (WaP)

Year	Origin	Release site	No. Koalas	Lat	Long	Nearest Locality	Reference number/s
1953	PI	Narrawong East	33	-38.224	141.793	Tyrendarra	4999532
1979	FI	Tower Hill	17	-38.34	142.376	Illowa	4999593
1981	FI	Ralph Illedge Sanctuary	14	-38.407	142.726	Naringal	4999583
1982	FI	Cooriemungle	25	-38.54	143.093	Cooriemungle	4999549
1982	FI	Timboon	36	-38.49	142.993	Timboon	4999592
1989	?	Orford	?	-38.209	142.036	St Helens	1053
1992	TH	Bessiebelle	29	-38.117	141.823	Homerton	23048
1994	TH	Bessiebelle	12	-38.123	141.869	Bessiebelle	23053
1994	TH	Kangaroobi Block	16	-38.615	143.167	Princetown	23054
1998	FR	Jancourt, Otways	72	-38.43	143.19	Jancourt East	61007
1998	FR	Simpson Kennedys Creek, Otways	25	-38.614	143.236	Kennedys Creek	61008
2002	ME	Bessiebelle State Forest	101	-38.205	141.851	Tyrendarra	79738-79805

Wimmera (Wim)

Year	Origin	Release site	No. Koalas	Lat	Long	Nearest Locality	Reference number/s
2002	ME	Beniagha Wildlife Reserve	13	-37.218	141.058	Poolaijelo	79732-79731