Addressing koala conservation management needs: applying novel genomic methods and assessing ecological exchangeability and genetic diversity across the species range.

Kjeldsen, Shannon Rikke

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Statement of the Contribution of Others

I was responsible for the production and integrity of all data presented here (unless otherwise stated), and all associated data filtering, management and interpretation for this project. I am the sole author for all chapters arising from this body of work, and the lead author in all associated publications. Specific co-author and collaborator contributions are outline in Table i.

Student and Supervisory Affiliations

	Role	Affiliation
Shannon Kjeldsen	PhD Candidate	a, b
Kyall Zenger	JCU Supervisor	a, b
Andrew Krockenberger	JCU Supervisor	c
Herman Raadsma	External Supervisor	d
Kellie Leigh	External Supervisor	e, f
Jennifer Tobey	External Supervisor	g

a) Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia
b) Centre for Tropical Bioinformatics and Molecular Biology, College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia
c) Centre for Tropical Environmental and Sustainability Science, James Cook University, Cairns, QLD 4878, Australia
d) Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camden, Private Mail Bag 4003, Narellan, NSW, 2570, Australia
e) Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, Camden, Private Mail Bag 4003, Narellan, NSW, 2570, Australia
f) Science for Wildlife, PO Box 286, Cammeray, NSW, 2062, Australia
g) San Diego Zoo Institute for Conservation Research, Escondido, CA, 92027, USA

Table i. Contributions of others

Chapter	Nature of Assistance	Name
1	Literature review, writing and editing	Shannon Kjeldsen
	Supervision and editing	Kyall Zenger, Herman Raadsma, Kellie Leigh, Jennifer Tobey, Andrew Krockenberger
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	Project conception, initial project design, funding, supervision, and manuscript editing	Kyall Zenger, Herman Raadsma, Kellie Leigh, Jennifer Tobey
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3	Execution and management of the project, including all data collection, protocol optimisation and analysis, writing, and editing	Shannon Kjeldsen
	Project conception, initial project design, funding, advice on data filtering and analysis, supervision, and editing	Kyall Zenger, Herman Raadsma, Kellie Leigh, Jennifer Tobey, Andrew Krockenberger
	Provision of samples and manuscript editing	Kellie Leigh, Jennifer Tobey, Damien Higgins, David Phalen, William Ellis, Emily Hynes
4	Project conception, project design, execution and management of the project, including all data collection, protocol optimisation and analysis, writing, and editing	Shannon Kjeldsen
	Project conception, initial project design, funding, supervision, and editing	Kyall Zenger, Herman Raadsma, Kellie Leigh, Jennifer Tobey, Andrew Krockenberger
	Advice on data filtering and analysis	Kyall Zenger, Kellie Leigh, Andrew Krockenberger, Catarina de Silva
5	Execution and management of the project, including all data collection, protocol optimisation and analysis, writing, and editing	Shannon Kjeldsen
	Project conception, initial project design, funding, supervision, and editing	Kyall Zenger, Herman Raadsma, Kellie Leigh, Jennifer Tobey, Andrew Krockenberger
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General Abstract

The Holocene epoch has been largely defined by the impacts of human activities on the environment. Anthropogenic processes have caused widespread environmental changes, leading to reduced biodiversity across the world. Habitat loss (or fragmentation), caused by large scale deforestation and urbanization, has contributed to mass extinction events in recent years. Global biodiversity is also threatened by a rapidly changing climate, resulting in increased frequency of major stochastic events. As with deforestation, these processes also result in habitat loss and fragmentation. Long term survival of any species relies on access to essential resources, i.e. food, water, shelter, and mates. The loss of habitat restricts access to these vital resources, limits movement, and ultimately jeopardises a species' ability to thrive.

The koala (*Phascolarctos cinereus*) is a recognised specialist folivore found across the East coast of Australia, and is found throughout a variety of habitat types (bioregions) and urban areas, from sub-tropical regions in northern Queensland, down through to temperate, and alpine regions in South Australia. The koala's natural range closely reflects the eucalyptus (and Acacia) species which are essential to its survival. Unfortunately, as with many other Australian animals, its long term survival is at risk from habitat loss through anthropogenic activities, disease and other major stochastic events (i.e. climate extremes and bushfires).

Despite their iconic status, there has previously been limited information on many key aspects of koala biology and ecology at a species-wide scale, including a dearth of critical data to assess population genetic diversity, divergence, and gene-flow across regions. Population genetic data is fundamental to prioritise current conservation efforts, and better inform future management decisions. One of the most effective ways to understand population structuring and large scale connectivity is through examining genetic signatures from animals across the species range. Previous koala genetic research has provided useful baseline information for koala populations, but typically at a small scale or a single point in time. Furthermore, these studies utilized different methods, and as such, cannot be directly compared with one another. To remedy this, comprehensive genetic data was collected across the entire species range, using a uniform genomic method is required to provide a solid foundation for all future studies. It is essential that this species-wide information is comprised from; different bio-regions, across different geographical scales, captures differences in biological traits and systems, and includes data from both wild and captive breeding populations.

This thesis focusses specifically on the development of genomic information which is essential for developing a deeper understanding of the ecology and biology of the koala, and presents an integrated approach which may be utilised in other vulnerable species. The primary objectives of this thesis are to: i) Provide insights into genetic diversity of koala populations across their natural and introduced range, using a novel genomic approach; ii) Investigate patterns of population structuring, connectivity,

and gene-flow between regions to suggest appropriate spatial scales for regional management of this species. iii) Provide insights into historical divergence among koala populations, clarifying questions surrounding speciation, and defining evolutionarily significant units (ESUs) for use in future legislation. iv) Assess relationships between biotic and abiotic factors which influence genetic structuring of koalas within and between regions across Australia, and to identify key environmental factors which may be relevant to koala management, and v) to develop a universal, accessible, and widely applicable genomic resource to aid in continued monitoring and conservation of koalas, to be used in testing for parentage, traceability, and routine diversity studies.

This project contains the first development and use of genome-wide markers (single nucleotide polymorphisms – SNPs) in the koala. Using the most comprehensive sampling range to date (n=800), I have used these novel resources to assess genetic diversity, and population differentiation at a species level, across regions and habitat types. The results of this species-wide survey indicate that most koala populations display levels of genomic diversity comparable to other outbred species, except for those populations impacted by population reductions. Koalas from the southern regions of Australia generally showed reduced genetic diversity at a state-wide level, which is likely a result of population declines as a result of hunting in the early 20th century. Genetic clustering analysis and phylogenetic reconstruction revealeda lack of support for current taxonomic classification of the three previous defined koala sub-species, with only a single ESU supported. Furthermore, ~70% of genetic variance is accounted for at the individual level, rather than between regional groups. The Sydney Basin region is highlighted as a unique reservoir of genetic diversity, having higher diversity levels (i.e. Blue Mountains region; $AvHe^{corr}$=0.20, PL%=68.6). This region appears to contain genomic signatures from across the species range, and as such should be highlighted as a key area for conservation efforts. Broad-scale population differentiation is primarily driven by an isolation-by-distance genetic structure model (49% of genetic variance), with some observed clinal local adaptation corresponding to habitat bioregions.

Signatures of selection were detected between bioregions (IBRA), with no single region returning evidence of strong selection. Environmental factors including habitat type, variation in rainfall and proximity to a national park (or protected area) explained a significant proportion of genetic variance between regions (R^2>0.99). When looking at geographic distance alone, spatial autocorrelation analysis indicated that at a distance of <5km, male and female relatedness were similar, although region-specific differences were observed beyond this distance. These differences were largely attributed to degree of habitat fragmentation in each region. Individual genetic relatedness within a 40-50km range correlated weakly with geographic distance in some regions, but other environmental factors and the effects of barriers to dispersal are more evident beyond this point. Factors influencing genetic population structure were variable between regions, however proximity to suitable habitat types was consistently linked to higher diversity levels.

To provide a universal genomic resource for ongoing monitoring of koala populations, a panel of 1,850 SNP markers (split across two distinct panels) were developed and tested, which were highly informative to estimate assign parentage, determine the source population of an individual koala across the entire species range (based on n=850), and estimate genetic diversity (based on n=1,000). This resource was developed using samples from across the species range, including a captive population, and will be used as an international reference panel for the majority of all ongoing data collection and genetic analyses as part of the national Koala management framework recovery program.

This body of work provides the fundamental baseline knowledge needed for the development of a comprehensive, data-driven national management strategy for koalas across Australia. Described here are the practical resources required for robust, continuing, and comparable monitoring of koala populations in the future. The overarching aim of the research outlined in this thesis is to encourage and facilitate evidence-based management of not only the koala, but also to provide a framework for development of these tools in other non-model species. By developing resources which are reliable, and widely accessible, I hope that this will encourage future collaborations, and lead to effective conservation of koalas in the future.

Table of Contents

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

List of Supplementary materials

All supplementary materials are available in the attached link:

https://www.dropbox.com/sh/azc5nmjf3fb50m0/AADCfNuhmHpeWK26yTsTBs__a?dl=0

Chapter 2

Supplementary data 2.1 Genotype file in the STRUCTURE format containing 3,060 SNP markers across all individuals. A value of '-9' indicates missing data

(Available via: https://link.springer.com/article/10.1007/s10592-015-0784-3#Sec21).

Chapter 3

Supplementary Figure 3.1. Population genetic distance tree constructed using a neighbour-joining, using Nei's (1978) unbiased genetic distances from Table 3.2.

Supplementary Figure 3.2. **a)** Maximum likelihood trees constructed using SNP markers, **b)** Bayesian tree constructed using PAV markers.

Supplementary Table 3.1. Hierarchical analysis of molecular variances between multiple groupings of populations based on NetView R clustering at various k-NN values. **a)** Northern clade and Southern clade, **b)** Four Groupings (1-[Magnetic Island, St Bees Island, St Lawrence, Maryborough, Moreton Bay, Koala Coast, Ipswich, Lismore], 2-[Woolgoolga, Port Macquarie], 3-[Gunnedah, Blue Mountains, Campbelltown, Southern Highlands], 4-[South Gippsland, Strzelecki, French Island, Cape Otway, Hamilton, Mt Lofty, Kangaroo Island]), **c)** Five Groups (based on *mtDNA* genetic divides proposed by Neaves *et al*. 2016), **d)** Ten groups (1-[Magnetic Island, St Bees Island, St Lawrence, 2-[Maryborough], 3-[Moreton Bay, Koala Coast, Ipswich], 4-[Lismore], 5-[Woolgoolga, Port Macquarie], 6-[Gunnedah], 7-[Blue Mountains, Campbelltown, Southern Highlands], 8-[South Gippsland, Strzelecki], 9-[French Island, Cape Otway, Hamilton], 10- [Mt Lofty, Kangaroo Island]).

Chapter 4

Supplementary Table 4.1 Environmental data for each sampling point.

Supplementary Table 4.2 Genomic relatedness metrics between each sampled individual.

Supplementary Figure 4.1 Correlation plot between environmental variables.

Chapter 5

Chapter 1: General Introduction

Background

The Holocene epoch has seen mass extinction events which are largely the result of human activity (Braje and Erlandson, 2013). Sometimes referred to as the "sixth great extinction", these events have resulted in the loss of species across all evolutionary domains (Braje and Erlandson, 2013). Anthropogenic processes have caused widespread environmental changes, with deforestation (and subsequent habitat loss) being a significant threat to biodiversity across the world (McDowell, 2014). Biodiversity is essential to maintaining the complex web of interactions that support a functional and productive ecosystem. All species, including humans, rely on these ecosystems in many ways, and so maintenance of biodiversity and management of species is imperative to ensure their survival.

Conservation of every species within an ecosystem would be extremely challenging, and is not economically viable at a large scale. The presence of cryptic or undiscovered species further complicates the effective management of whole ecosystems, as little to no information is available for these species (Piggott and Taylor, 2003). Accordingly, government or conservation resources are frequently allocated to high profile or "desirable" species, with many other species disregarded irrespective of their importance to the ecosystem as a whole. However, focusing resources on a single charismatic species within a complex ecosystem can lead to broader positive ecological outcomes, with many other species being passively managed (Hausmann *et al*. 2017, Rodrigues and Brooks, 2007, Roberge and Angelstam, 2004).

The Australian continent has been physically isolated for between 35 and 46 million years (MY/MYA; Wroe, 1999), and as such is now home to ancient lineages of species not present throughout the rest of the world. It is one of the most biologically diverse countries in the world, with many endemic species being unique to Australia (Wroe, 1999). The Australian continent spans more than 33 degrees of latitude below the equator, in line with the Tropic of Capricorn, and contains a variety of climate zones, from tropical to alpine. This variation allows for high levels of habitat diversity across the country, with a total of 89 different bioregions (Thackway and Cresswell, 1997). Australia boasts the largest number of native marsupial species, and the only two extant members of the monotreme family (Nilsson *et al.* 2004). Despite this, Australia has some of the highest levels of deforestation when compared to other developed nations, which threatens many of these unique species (Bradshaw, 2012). Since European colonization, Australia has lost more than 100 vertebrate species to extinction (recorded) (Woinarski *et al*. 2019). With a predicted increase in climate extremes, an increase in the frequency of catastrophic events (e.g. floods, droughts, and bushfires), and an increase in land clearing (in New South Wales specifically), the number of species threatened with extinction is set to rise rapidly over the coming years. Both climate change, and other anthropogenic impacts are likely to lead to lower habitat diversity, meaning that species will need to adapt to new environments to survive (Ravigné *et al.* 2009). While

species which are considered generalists may achieve this with ease, species which have specialist biological requirements will be at risk (Clavel *et al.* 2011). Understanding the biology, population structure, and threatening processes of these species will allow for a more targeted approach to management and conservation.

Every species has a number of essential resources which are required for survival (both long and short term). Generally, these fall into three overarching categories – (1) the need for sustenance (food and water), (2) the need for shelter, and (3) the ability to reproduce (Wilkin and Gray-Wilson, 2015). A species will ultimately be successful if all these needs are met, and other factors such as disease, and excessive predation, are limited. Within each of these categories, every species will have a degree of specificity. For example, organisms which are only capable of feeding on, and creating nests in a single species of tree may have less interspecific competition for resources, and would be considered to be a highly specialised species. However, if this tree species is lost, the specialist organism will lose its niche, and become extinct. Whereas if the organism is able to utilise many species of tree for food and to build its nests, it will be less sensitive to changes in its environment, and would be considered to be a more generalist species. These evolutionary approaches are a trade-off between reducing interspecific competition for resources, and becoming more vulnerable to stochastic changes in the environment (Matthews *et al.* 2014, Pflüger *et al.* 2019).

In a world where anthropogenic processes have significantly altered natural environments, generalist species have come to thrive in urban and modified habitats, as they are able to take advantage of a wide range of environmental niches. An example of an Australian generalist species is the white ibis (*Threskiornis Molucca*). The Australian white ibis has an elongated, curved beak, specialised for foraging in wetlands (Martin *et al.* 2010). While its beak, and featherless head may indicate a speciality for a wetland environment, this species has now become common in urban areas. Its beak has enabled it to scavenge for food in cities – eating a variety of discarded human food, insects and carrion from rubbish bins, which has led to the white ibis being affectionately known to many Australians as the "Bin Chicken". While its natural habitat may be threatened, the Australian white ibis has successfully adapted to fill a new niche in the urban environment (Martin *et al.* 2010). However, many other species have not been able to similarly adapt to changes in their environments, and these populations have progressively become more fragmented over time. Loss of habitat has led to the decline in many species with specific dietary, reproductive, or habitat requirements.

Arguably, Australia's most widely recognized and charismatic species groups are the marsupials. This group diverged from other lineages during the late Cretaceous era, with more than 300 extant species distributed across the world (Mitchell *et al.* 2014a). Australia is home to the majority of these species, with many of these being the only surviving species in their respective taxonomic families (Figure 1.1). These animals have adapted to a wide variety of different habitats and climates, with many being highly

adapted to climate extremes, such as extended periods of drought (e.g. Koalas). Similarly, many marsupials of the same species group are distributed across vastly different habitats and climates, making them ideal candidate species to help focus research and conservation efforts (and facilitate passive conservation of other species). Two such species groups are the kangaroo (Macropodidae) and the koala (Phascolarctidae). Conservation efforts using a targeted species approach requires a comprehensive and integrated knowledge of both their biology and ecology (Rodrigues and Brooks, 2007). Establishing even baseline resources for non-model organisms such as these can be extremely challenging; these research projects can be logistically difficult (e.g. regarding the complex politics of working with a charismatic species, difficulties in sampling, and access to technologies), and the associated costs are often prohibitive in a conservation setting (Naidoo *et al.* 2006). As such, ensuring that resources are used efficiently is paramount to their success. The family Macropodidae consist of many distinct species, with different biological needs and ecological histories, meaning a focus on this family would result in the need for increased resources. The family Phascolarctidae however, has a single extant species, the koala, which allows for a more targeted research effort.

Figure 1.1. Abridged view of marsupial species phylogeny, including Australian members of the families Dasyuridae, Macropodidae, Vombatidae, and Phascolarctidae, adapted from (Mitchell *et al.* 2014b). Boxed in red are the only extant species of the family Phascolarctidae (koalas), and its closest living relatives; wombats.

The Koala

Biology and ecology of the koala

The koala (*Phascolarctos cinereus* (Goldfuss 1817) – Diprotodontia, Marsupialia) is currently one of Australia's most charismatic and iconic species. Koalas are folivorous aboreal marsupials, and are the only extant members of the family Phascolarctidae (Box 1.1; Figure 1.1).

Koalas are distributed along the east coast of Australia, and are present in a variety of habitat types, ranging from sub-alpine forests in Victoria, to the sub-tropical forests in far north Queensland (Phillips, 2000). The koala exhibits phenotypic differences between its northernmost and southernmost populations (Briscoe *et al.* 2015), with clinal variation tending towards larger individuals, with more variation in coat colour and increased coat length in the southern regions (Woinarski and Burbidge, 2020).

In the past, it has been widely accepted that koalas may be divided into three ecologically significant units (ESUs) or three allopatric sub-species; *Phascolarctos cinereus adustus* (Thomas, 1923), *Phascolarctos cinereus cinereus* (Troughton 1941) and *Phascolarctos cinereus victor* (Troughton, 1935). These divisions were characterized in the first half of the 20th century from a small number of skins and skull specimens (Houlden *et al.* 1999). The geographic borders separating the subspecies have never been explicitly defined and it is often accepted that the subspecies be defined by state borders (Natural Resource Management Ministerial Council 2009). Taxonomic distinction of the koala has been an extremely controversial topic and has historically been defined based solely on morphological information (Houlden *et al.* 1999). Subspecies definitions are now more reliant on biological compatibility and genetic distances, but surprisingly, this had yet to be definitively investigated in the koala at the beginning of this thesis. All genetic research to date indicates that differences in the koala across its range can be attributed to clinal variation or phenotypic plasticity, rather than a process of speciation (Houlden *et al.* 1999, Briscoe *et al.* 2015). Analysis of the koala's mitochondrial genome (*mtDNA*) indicates a lack of support for subspecies of koalas, however until recently, sampling in these studies was sparse, with large distances between sampling populations, and did not cover the entire species range (Houlden *et al.* 1999, Houlden *et al.* 1996b, Tsangaras *et al.* 2012).

Despite their broad geographic/climatic range, koalas are considered to be a dietary specialist, and as such feed solely on leaves from eucalyptus and acacia families. Koalas have been found to utilise up to 120 species of dietary trees across their species range, however individual preferences differ in each region, with primary food tree species being as few as two in any given region (Melzer *et al.* 2000, Tucker *et al.* 2008). Interestingly, these preferences appear to be closely linked to maternal preferences, suggesting that individual tree selection may be learned (Tucker *et al.* 2008).

Previous studies examining dispersal patterns in koalas have shown that home ranges are variable, but are rarely larger than 40 hectares (White, 1994, Adams‐Hosking *et al.* 2012). While dispersal has been shown to differ between sexes, generally the size of an individual's home range will depend on the availability of resources; i.e. food and mates. Sub-adult males are more likely to travel longer distances to establish new territory (and mates), whereas females will generally remain closer to their place of birth. Koalas are polygamous, and despite a dominant male often being present within a given territory, studies have indicated that there is no more likelihood of the dominant male siring offspring compared to transient males (Gordon *et al.* 1990, White, 1994). Similarly, no evidence of inbreeding avoidance has been observed in the koala, which presents concerns if populations are physically isolated, as inbreeding may increase more rapidly over time in small, isolated populations (Schultz, 2019).

Box 0.1 Summarised from (Martin and Handasyde, 1999)

Life history traits
Phascolarctos cinereus (Goldfuss 1817) – Diprotodontia, Marsupialia

Body weight/Size:

Northern females (4.1-7.3kg), Northern males (4.2-9.1kg)

Southern females (7-11kg). Southern males (9.5-14.9kg)

Phenotypic characteristics:

Light to dark grey coat colour, short length (Northern, warm climates)

Light to dark grey, light to dark, reddish brown coat colour, medium to long length (Southern, cool climates)

Life span:

Average of 12-14 years (up to 18-20 years)

35 day gestation, 6-8 months in pouch, 12 months independent, 2 years sexually mature

Historic range/current range

The first fossil records from the family Phascolarctidae emerge during the late Oligocene (26-23.5 MYA), and have been found largely along the east coast of the Australian continent. Fossils have been found dating from between the late Oligocene and the late-Pleistocene, spanning much of the southern regions of Australia, even within south-eastern Western Australia (Shabani *et al.* 2019). These fossils belong to five genera (*Madakoala*, *Periloala*, *Nimiokoala*, *Litokoala* and *Phascolarctos*), with *Phascolarctos* being the only surviving genus beyond the Miocene era (Louys *et al.* 2007).

Changes in climate since the last glacial maximum (~19-23 ka [kiloannum]) have led to range shifts of suitable koala habitat. While these shifts created significant barriers to gene flow (e.g. Hunter valley rift), many of these barriers had once again receded by the early-mid Holocene (~5-7 ka) (Shabani *et*

al. 2019). In other Australian species, key barriers to dispersal along the east coastline include the Great Dividing Range, and the major river systems which diverge from the Murray-Darling Basin. These barriers have resulted in fragmentation in several species (Bryant and Krosch, 2016), including ancient assassin spiders (Archaeidae; Rix and Harvey, 2012), garden skinks (Scincidae; Chapple *et al.* 2011), giant burrowing frogs (*Heleioporus australiacus*; Penman *et al.* 2005), common froglets (*Crinia signifera*; Symula *et al.* 2008), eastern yellow robins (*Eopsaltria australis*; Morales *et al.* 2015) and brush-tailed rock wallabies (*Petrogale penicillata*; Hazlitt *et al.* 2014). During range expansions during glacial minimums, barriers to dispersal may have been reduced, allowing for more connectivity to inland populations, and between northern and southern regions. Fossil records indicate that koalas were present in much of inland Australia, where they are not seen today (Adams-Hosking *et al.* 2011a, Adams-Hosking *et al.* 2011b, Bryant and Krosch, 2016).

Threats and conservation concerns

Despite their iconic status, the koala has been challenged with a variety of threats throughout its history; both natural, in the form of chlamydial and retroviral infection, and anthropogenic, through hunting, predation by feral species and habitat loss (Adams-Hosking *et al.* 2011b, Melzer *et al.* 2000, Menkhorst, 2008, Martin and Handasyde, 1999).

Prior to European settlement, the koala was widespread across Eastern Australia, and held cultural significance to many indigenous groups across Australia (Schlagloth *et al.* 2018). Though they were hunted for their meat, many tribes had strict customs relating to how they may be hunted and eaten. Koala skins were not to be removed, and bones were not to be broken when koalas were hunted. These traditions were linked to dreamtime stories which threatened severe drought if these laws were not followed (Schlagloth *et al.* 2018). Evidence suggests that indigenous hunting did not cause a significant decline in koala numbers, however changes in land management (specifically regular vegetation burning) after indigenous migration (~50,000 years ago) may have altered landscapes and changed the overall range of the koala in Australia (Bowman, 2003, Vigilante and Bowman, 2004).

Following European settlement, koala numbers dramatically declined as a result of widespread hunting. Koalas were hunted primarily for their pelts, and this resulted in a significant reduction in numbers across the species range, and many local extinctions, particularly in the southern regions (Menkhorst, 2008). Hunting of koalas in the northern regions of Australia was not as widespread, and many populations remained relatively untouched during this period. By the early 20th century, as few as 1,000 individuals remained in Victoria, and natural koala populations were extinct in South Australia (Melzer *et al.* 2000, Menkhorst, 2008, Gordon and Hrdina, 2005, Gordon *et al.* 2006, Hrdina and Gordon, 2004).

Following widespread population crashes, the koala was listed as a protected species in the 1930's. All hunting for pelts was banned, and trade of koala products was heavily restricted. A re-introduction program over the following 75 years has since re-established koala numbers in these existing and new

southern populations (Menkhorst, 2004). Although southern population numbers have now largely recovered, most re-introductions were sourced from introduced island populations, which limited genetic diversity in these areas.

Though overall numbers of koalas are believed to be declining steadily (Woinarski and Burbidge, 2020), current studies indicate high variability in regional numbers and in genetic structuring across the species' range (Melzer *et al.* 2000, Menkhorst, 2008). Habitat fragmentation and loss due to land clearing are the primary threats to koala survival across Australia, followed closely by threats from introduced species (e.g. feral dogs) (Gentle *et al.* 2019) and disease (chlamydia and koala retrovirus) (Melzer *et al.* 2000, Menkhorst, 2008). However, while numbers decline in the northern regions, southern populations have become overabundant in many areas (Menkhorst *et al.* 2019). This overabundance has resulted in food trees being defoliated, leading to starvation of koalas, and requiring subsequent active management of population numbers by local governments (i.e. culling) (Menkhorst *et al.* 2019).

This regional variation has made management of koala populations difficult at a national scale. Even within states, matters are complicated by local variations in habitat, land use, and disease status. Under Australian federal law (and under the IUCN red list), the koala is classified as "Vulnerable to extinction" (Woinarski and Burbidge, 2020). Koalas are also listed as a threatened species under the US Endangered Species Act (Fish and Wildlife Service 1998; Natural Resource Management Ministerial Council 2009). Despite being such an iconic species, surprisingly few studies have attempted to evaluate broad-scale population connectivity and structure across divergent landscapes. Most studies have been confined to a local scale, leading to a dearth of information at the species level (Kjeldsen *et al.* 2016, Kjeldsen *et al.* 2019).

Specialist species, like the koala, are heavily reliant on ecological niches, and so habitat fragmentation has a more pronounced effect on population connectivity (when compared to more generalist species) (Adams‐Hosking *et al.* 2012, Matthews *et al.* 2014, Pflüger *et al.* 2019). Despite the broad distribution of koalas across different bioregions and climates, the koala's specialised dietary requirements still need to be met. Understanding how habitat continuity influence behaviour and subsequent gene flow in the koala is vital to developing effective conservation plans.

Importance of genetic resources

Successful management of any wild species relies on a thorough understanding of population structuring and connectivity across the species range. Patterns of structuring may also differ between regions, and it is important to incorporate this knowledge into management strategies (Adams‐Hosking *et al.* 2012, Margules and Pressey, 2000, Rodrigues and Brooks, 2007). The study of animal genetics allows us to obtain information on many scales, from fine-scale relationships between

individuals in a population or family (e.g. parent-offspring assignments), to relationships between individuals at the most distant extents of the species range. Genetic analysis can even allow us to investigate the process of speciation (Coyne, 1992, Schluter and Conte, 2009, Orr, 1995).

Until recently, species specific genetic resources, such as genome-wide genetic markers were difficult to develop. The costs of development, and a lack of existing resources, meant that they were not frequently utilized outside of human or agricultural settings. More recently, with the development of next generation sequencing and new genomic sampling techniques, genetic resources have become more cost effective to develop. This has allowed for the rapid incorporation of genetic information into management strategies of non-model organisms.

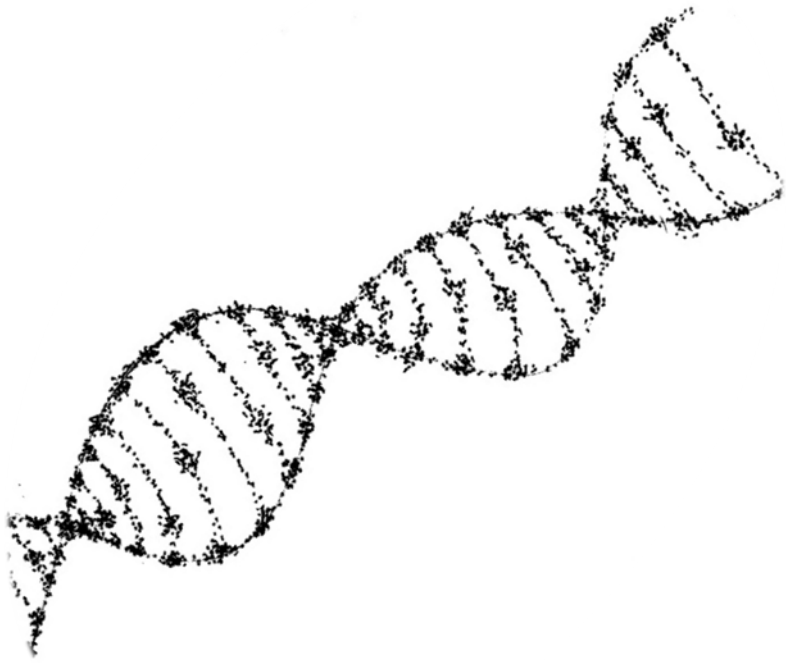
Several genetic studies have attempted to unravel population structuring and relatedness in the koala. These studies were mostly conducted on a local scale which limited their interpretation across the species distribution (Schultz *et al.* 2020, Wedrowicz *et al.* 2019, Wedrowicz *et al.* 2018, Wedrowicz *et al.* 2013, Fowler *et al.* 2000, Houlden *et al.* 1999, Houlden *et al.* 1996b, Lee *et al.* 2010a, Lee *et al.* 2012a, Neaves *et al.* 2016, Timms *et al.* 1993, Wilmer *et al.* 1993). Most previous studies have also assessed populations with large gaps between sampling regions (eg. Houlden *et al.* 1996; Wedrowicz *et al.* 2018), and so were not able to detect gradual changes within and between populations. Additionally, most studies to date have utilised tradition markers, including minisatellites (Taylor *et al.* 1991), microsatellites (Houlden *et al.* 1996a, Houlden *et al.* 1996b, Lee *et al.* 2012a, Ruiz-Rodriguez *et al.* 2014, Wedrowicz *et al.* 2018), *mtDNA* (Houlden *et al.* 1999, Taylor *et al.* 1997, Tsangaras *et al.* 2012, Wedrowicz *et al.* 2018) and major histocompatibility complex (MHC) genes (Houlden *et al.* 1996d, Houlden *et al.* 1996c, Jobbins *et al.* 2012, Lau *et al.* 2014).

Traditional genetic markers can provide valuable insights into genetic diversity and divergence of a species. However, their use needs to be carefully matched to specific research questions, with data interpretations also potentially limited by the lower number of loci typically selected. Mitochondrial DNA markers are extremely useful for examining phylogenetic relationships between species and divergent populations, as *mtDNA* tends to be conserved over an evolutionary time frame. Unfortunately, this characteristic also makes detecting fine-scale differences within and between populations (particularly at a regional scale) difficult. Furthermore, *mtDNA* is a single locus, which is maternally inherited, and as such does not fully resolve contemporary patterns of genome-wide micro-evolutionary processes. For example, an early study by Houlden *et al.* (1999) described the phylogenetic relationship between koala populations across Australia using the mitochondrial control region. This study examined samples from a large proportion of the koala's natural and introduced range, but was unable to identify any variation within populations. These results are similar to other studies using *mtDNA* (Houlden *et al.* 1999, Neaves *et al.* 2016, Taylor *et al.* 1997, Tsangaras *et al.* 2012, Wedrowicz *et al.* 2018), which also indicated low diversity using this type of genetic marker.

Studies which utilise microsatellite markers are largely able to overcome fine-scale genetic data resolution issues, as they are inherently selected for their high levels of variation between individuals. This allows for evaluation of familial relationships (ie. Parent-offspring groups), and detection of gene flow between populations. Microsatellite markers generally outperform *mtDNA* for fine-scale resolution, but they are incompatible for phylogenetic analyses (due to fast evolution rate) and can also be error-prone due to their hypervariable nature. Inconsistent allele calling can lead to skewed diversity and divergence estimates, making comparisons between studies difficult. These errors become more pronounced as more markers are used, so there is often a trade-off between accuracy and overall statistical power with this type of marker (DeFaveri *et al.* 2013; Miller *et al.* 2014; Rasic *et al.* 2014). In general, increasing the overall number of markers used gives more statistical power to assess patterns of diversity, divergence and adaptive variation (Angeloni *et al.* 2012; Davey *et al.* 2011). However, increasing the number of microsatellites substantially increases overall project costs and time with limited analytical utility compared to more modern types of genome-wide markers (Guppy *et al.* 2018).

Genome-wide markers, such as SNPs, are highly abundant throughout the genome, and so provide an alternative option to more traditional approaches. SNPs are bi-allelic, and are (per marker) less informative than their multi-allelic equivalents (eg. Microsatellites). However, SNPs can be identified at a high genomic density, within both genic and intergenic regions, and require no prior genomic resources for development (DeFaveri *et al.* 2013; Vignal *et al.* 2002). This makes them a more cost-effective and versatile option for genomic studies (Guppy *et al.* 2018). Studies utilizing traditional genetic markers certainly provided an important foundational understanding of koala diversity and divergence, however the genetic markers used previously are not genome-wide, and accordingly are unable to detect fine-scale population sub-structuring or genome-wide signatures of adaptive variation, and so until recently, this had not been examined in the koala (Kjeldsen *et al.* 2016). While koalas from across the species' distribution may successfully breed and produce viable offspring, the significant phenotypic differences between regions suggest that local adaptation may be occurring (Briscoe *et al.* 2015). If so, at what level do we manage a species potentially defined as a single ESU, which has such a wide geographic and climatic range? While previous research has been extremely helpful to create baseline information on koalas, these studies rarely sampled across regions, and used a variety of traditional genetic methods, which made comparisons between studies difficult, if not impossible. Comprehensive sampling across the entire species range, and the use of a single genomic method could provide a comprehensive foundation for all future studies. The development of a universal genomic tool would standardize new information, and allow for comparisons between regions, and across time scales. In addition, the integration of genomic data with environmental, and behavioral data will provide a comprehensive, holistic knowledge base in this species. This in turn will lead to better management of koalas across their distribution, conserving important habitat, and ultimately, aiding in the conservation of the ecosystems that koalas inhabit.

This thesis provides comprehensive information on taxonomic classification, population structuring, connectivity and diversity of koalas across their distribution. In addition, it has developed novel genomic resources, and has applied these to investigate local adaptation within and between regions, and to provide accessible, standardized genomic resources so that future studies may be directly comparable. This resource aims to encourage and facilitate evidence-based management of koala populations across Australia, and to provide a framework for development of these tools in other non-model species.



Chapter 2: Development of novel genomic resources in the koala

Genome-wide SNP loci reveal novel insights into koala (*Phascolarctos cinereus*) population variability across its range

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Research Objectives and Chapter Summary

Background/Gap in knowledge

Koalas (*Phascolarctos cinereus*) are one of Australia's most widely known and iconic marsupial species, found across the east coast of Australia from northern Queensland through to South Australia.

Successful management is reliant on identifying the genetic structuring within and between populations, and identifying the genomic potential to adapt to different environments, particularly to inform captive breeding strategies.

With an increase in the availability and affordability of next-generation sequencing technologies, genome-wide markers are becoming increasingly popular in evolutionary and ecological research.

Aims

(i) Assess the viability of ddRAD/RADseq as a tool to rapidly discover SNP markers in this species,

(ii) Assess this method to look at levels of genome-wide variation within animals from eight geographically distinct regions from across the species range,

(iii) Assess the potential utility of the SNP markers identified to answer a broad range of ecological questions, including broad- and fine-scale population structuring, identifying signatures of selection, and identify sex-linked markers.

Significance/Conclusions

The results outlined in this study have provided the first insights into genome-wide diversity in koala populations across their range.

This is the first use of genome-wide markers to assess population differentiation at a broad-scale in the koala and the first time that sex linked SNPs have been identified in this species. The application of this novel genomic resource to populations across the species range will provide in-depth information allowing informed conservation priorities and management plans for *in situ* koalas across Australia and *ex situ* around the world.

Overall diversity within populations was revealed to be equivalent to other vertebrate species, including canid, felid and ungulate species.

Abstract

The koala (*Phascolarctos cinereus*) is an iconic Australian species that is currently undergoing a number of threatening processes, including disease and habitat loss. A thorough understanding of population genetic structuring and genomic variability of this species is essential to effectively manage populations across the species range. Using a reduced representation genome sequencing method known as double digest restriction-associated (ddRAD) sequencing, this study has provided the first genome-wide SNP marker panel in the koala. In this study, 33,019 loci were identified in the koala and a filtered panel of 3,060 high-utility SNP markers, including 95 sex-linked markers, were used to provide key insights into population variability and genomic variation in 171 koalas from eight populations across their geographic range. Broad-scale genetic differentiation between geographically separated populations (including sub-species) was assessed and revealed significant differentiation between all populations (F_{ST} range = 0.01 – 0.28), with the largest divergence observed between the three geographically distant subgroups (QLD, NSW and VIC) along the east coast of Australia (average F_{ST} range = 0.17 – 0.23). Sub-group divergence appears to be a reflection of an isolation by distance effect and sampling strategy rather than true evidence of sub-speciation. This is further supported by low proportions of AMOVA variation between sub-species groups (11.19%). Fine-scale analysis using genome-wide SNP loci and the NETVIEW pipeline revealed cryptic genetic sub-structuring within localised geographic regions, which corresponded to the hierarchical mating system of the species. High levels of genome-wide SNP heterozygosity were observed amongst all populations (He = 0.25 – 0.35), and when evaluating across the species to other vertebrate taxa were amongst the highest values observed. This illustrates that the species as a whole still retains high levels of diversity which is comparable to other outbred vertebrate taxa for genome-wide SNPs. Insights into the potential for adaptive variation in the koala were also gained using outlier analysis of genome-wide SNPs. A total of 48 putative outlier SNPs were identified indicating the high likelihood of local adaptations within populations and regions. This is the first use of genome-wide markers to assess population differentiation at a broad-scale in the koala and the first time that sex-linked SNPs have been identified in this species. The application of this novel genomic resource to populations across the species range will provide in-depth information allowing informed conservation priorities and management plans for in-situ koalas across Australia and ex-situ around the world.

Introduction

Koalas (*Phascolarctos cinereus)* are one of Australia's most widely known and iconic marsupial species, found across the east coast of Australia from northern Queensland through to South Australia (Figure 2.1). Koalas have faced a number of different conservation challenges throughout their history, from hunting for pelts, wide-spread disease including chlamydial infection and koala retrovirus (KoRV) and anthropogenic factors which led to habitat loss and predation by feral species (Avila-Arcos *et al.* 2013; Gordon *et al.* 2006; Melzer *et al.* 2000; Menkhorst 2008). There have been several documented events of local extinctions, range contractions, expansions and translocations throughout recent history. One of the greatest historical impact on the species was hunting in the early 20th century (Adams-Hosking *et al.* 2011; Gordon *et al.* 2006; Melzer *et al.* 2000; Menkhorst 2008). Despite numbers recovering well following extensive hunting, more recent loss of habitat, increasing urbanisation and disease have led to the koala being classified as vulnerable across most of its range (NSW, ACT and QLD) by the Federal Australian government, and listed as a threatened species under the US Endangered Species Act (Fish and Wildlife Service 1998; Natural Resource Management Ministerial Council 2009). Successful management is reliant on identifying the genetic structuring within and between populations, and identifying the genomic potential to adapt to different environments, particularly to inform captive breeding strategies (Moritz *et al.* 1996; Whisson and Carlyon, 2010; Whisson *et al.* 2012). Information regarding fine-scale genetic relatedness within a population can also provide insights into social structuring of individuals within a region (Ross & Fletcher 1985). This information is particularly important in species where one or both sexes have a defined home range with a hierarchical social structure, as is the case with koalas (Mitchell 1990a; Thompson 2006; Ellis *et al.* 2009).

Management of the koala has historically been a contentious issue as preliminary studies indicate high variability in population health and genetic structuring across Australia (Melzer *et al.* 2000). While in some regions koalas appear locally abundant, research indicates that numbers are generally declining across Australia (Gordon *et al.* 2006; Melzer *et al.* 2000; Natural Resource Management Ministerial Council 2009) and that local extinctions may currently be occurring (Lunney *et al.* 2002; Lunney *et al.* 2014).

Previous population genetic studies have utilised neutral genetic markers, including minisatellites (Taylor *et al.* 1991), microsatellites (Houlden *et al.* 1996a; Houlden *et al.* 1996b; Lee *et al.* 2012a; Ruiz-Rodriguez *et al.* 2014), *mtDNA* (Houlden *et al.* 1999; Taylor *et al.* 1997; Tsangaras *et al.* 2012) and MHC genes (Houlden *et al.* 1996c; Lau *et al.* 2013), but have often had difficulty defining genetic structuring, especially on a national scale. To date, genetic studies on the koala have used at most 14 markers to estimate diversity and divergence within and between populations and this research has been limited to studies at a regional scale (Ellis *et al.* 2002; Lee *et al.* 2012a). A study by Houlden *et al.* (1996) using six neutral markers, along with an assortment of other population genetic studies and

anecdotal evidence (Houlden *et al.* 1996b; Lee *et al.* 2010; Lee *et al.* 2012; Martin 1985; Seabrook *et al.* 2011; Seabrook *et al.* 2002; Sherwin *et al.* 2000; Smith & Smith 1990; Wilmer *et al.* 1993) indicated a general decline in koala numbers and a reduction in genetic diversity. Other studies have suggested low diversity at a species level due to several significant bottlenecks and inbreeding, particularly in island populations (Fowler *et al.* 2000; Houlden *et al.* 1996b; Houlden *et al.* 1996c; Lee *et al.* 2012a; Tsangaras *et al.* 2012; Wilmer *et al.* 1993). Recent research by Lee *et al.* (2010) specifically noted a strong decline in genetic diversity in the south eastern Queensland region. Likewise, studies looking at MHC diversity in Victorian koalas have also noted significant founder effects due to translocations from bottlenecked populations (Lau *et al.* 2014). However, there is no baseline for genetic diversity in the koala as levels of genetic variation prior to European settlement are largely unknown (Houlden *et al.* 1996).

No studies have assessed the genomic health of koalas at a national scale (Lee *et al.* 2012a). Based on morphological and geographical information, koalas have been grouped into three sub-species, loosely corresponding to the state political borders along the east coast of Australia (Natural Resource Management Ministerial Council 2009). This classification was largely based on skull morphology and general phenotype (size and fur colour), which vary greatly across the species range. However, genetic studies carried out since the grouping was established did not detect sufficient levels of differentiation for a sub-species classification (Houlden *et al.* 1999). In other species, such as trout and wolf-like canids, genome-wide markers have been used successfully to assess both broad-scale population structuring and speciation and have consistently been shown to provide accurate assessments of species divergence (Pollinger *et al.* 2011; Stephens *et al.* 2009). In the koala however, broad-scale genetic structuring across the species range using genome-wide markers has not been investigated.

With an increase in the availability and affordability of next-generation sequencing technologies, genome-wide markers are becoming increasingly popular in evolutionary and ecological research. These genotyping by sequencing (GBS) methods allow for unprecedented ease of research into non-model organisms. Many studies have indicated that the resolution provided by traditional markers such as allozymes, mini/microsatellites and mitochondrial DNA (*mtDNA*) is not comparable with their genome-wide equivalents (DeFaveri *et al.* 2013; Miller *et al.* 2014; Rasic *et al.* 2014). Markers with a higher genomic density can provide more comprehensive genomic information enabling detailed studies of general diversity, divergence and adaptive variation (Angeloni *et al.* 2012; Davey *et al.* 2011). Bi-allelic single nucleotide polymorphism (SNP) markers are less informative per marker than microsatellites, but have the advantage of being highly abundant across the genome and are theoretically evenly spread across the genome (DeFaveri *et al.* 2013; Vignal *et al.* 2002) on coding and non-coding loci. Using complexity reduction methods such as double digest restriction-associated DNA sequencing (ddRAD) (Peterson *et al.* 2012), many thousands of SNPs can be identified for the same cost as developing only a few microsatellites, making them an attractive alternative to traditional marker sets

(Peterson *et al.* 2012). The SNPs may also be present in both coding and non-coding regions of the genome and since many of these are likely to fall on the sex chromosomes (Carmichael *et al.* 2013), they can also be informative for research concerning sex-biased dispersal and sex-linked traits.

Genome-wide SNP genotyping is now considered the preferred marker for population based diversity studies in many species, and can be a versatile tool to provide insights into genetic structuring and the micro-evolutionary processes in the koala. These markers can be used to address critical questions for the species, including assessments of speciation (Jones *et al.* 2012; Leache *et al.* 2014), inter-regional and intra-regional population diversity and relatedness (Johnston *et al.* 2014; Larson *et al.* 2014), inbreeding, signatures of population reductions, effective populations sizes (Ne) (Johnston *et al.* 2014), parentage (Fernández *et al.* 2013), and evidence of adaptive variation (Nielsen *et al.* 2005). In this study, the development of the first panel of genome-wide SNP markers in the koala is reported, and will serve as an important tool for the conservation of the species and can be used to provide insights into population structuring and variability across the species range. This study assessed i) the viability of ddRAD as a tool to rapidly discover SNP markers in this species, ii) levels of genome-wide variation within animals from eight geographically distinct regions from across the species range, and iii) the potential utility of the SNP markers identified here to answer a broad range of ecological questions, including broad- and fine-scale population structuring.

Materials and Methods

Sampling and DNA extraction

To evaluate genetic differentiation amongst geographically distant or potentially bottlenecked populations using genome-wide SNP loci, blood and tissues samples were obtained from koalas in representative regions of Queensland, New South Wales and Victoria, along with one introduced population on St Bees Island, QLD (see Figure 2.1 for sampling locations). Tissue samples were opportunistically obtained from William Ellis, Sean FitzGibbon and Alistair Melzer and other researchers (see acknowledgements) and preserved in 70% ethanol, while whole-blood samples were stored at -20 °C. To ensure the highest quality of DNA for ddRAD library preparation, all DNA samples were extracted using a modified CTAB / Cholorform-Isoamyl method (Adamkewicz & Harasewych 1996) and further purified using Sephadex G-50 approach (GE Healthcare Life Sciences 2000) to ensure no inhibitors were carried through to ddRAD library preparation.

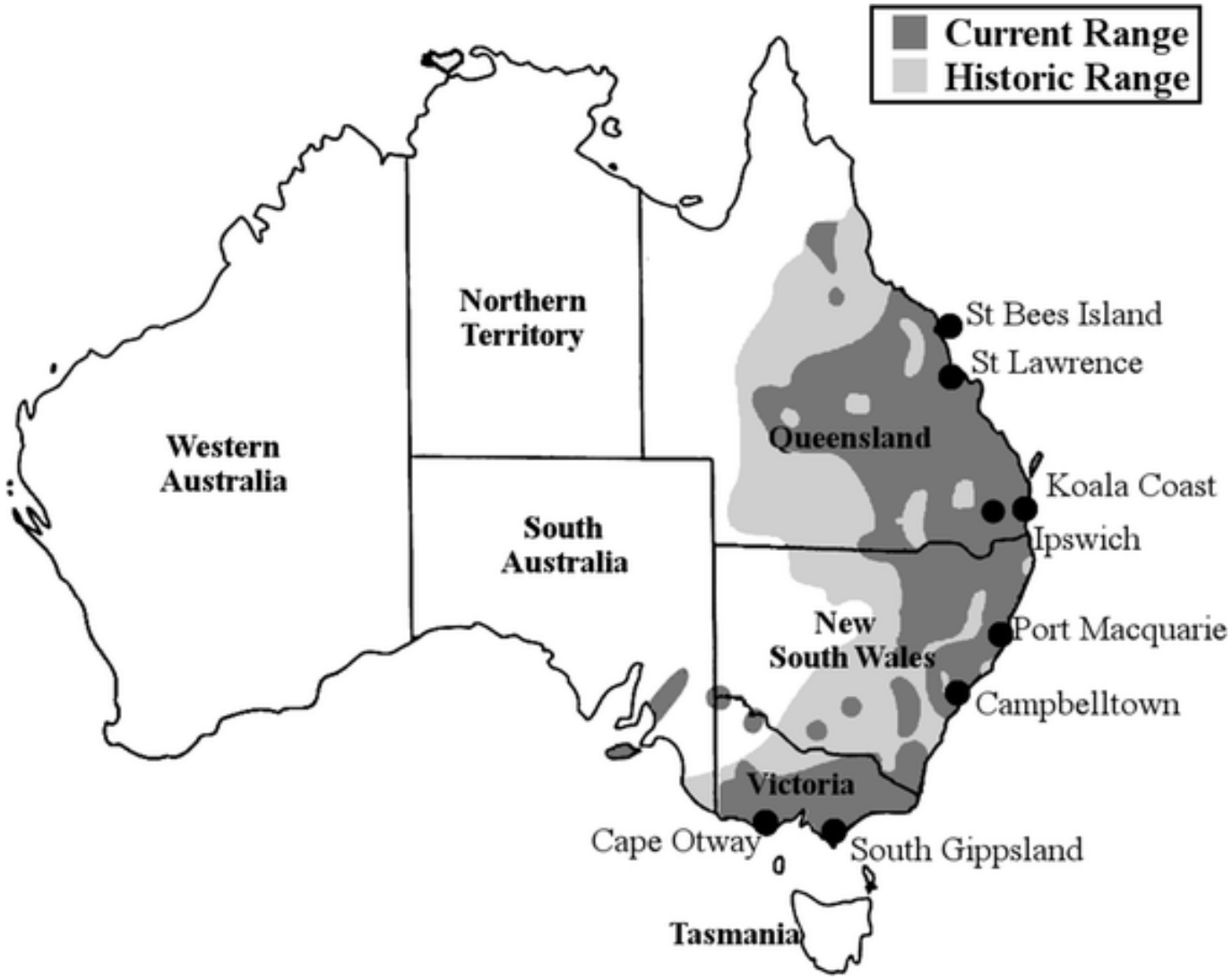

Figure 2.1. Koala Distribution Map: Current national distribution of koalas and historical range with sampling sites indicated. Adapted from distribution map created by Strahan *et al.* (1995)

ddRAD Library Preparation

In silico simulations of *EcoRI* and *MspI* double digests were first performed on the Tamar wallaby (*Macropus eugenii*) and Opossum (*Monodelphis domestica*) genomes and extrapolated to the equivalent koala genome size, to evaluate the effectiveness of these restriction enzymes on reducing the complexity of the koala genome for ddRAD library preparation (Peterson *et al.* 2012). Simulations were carried out using the SimRAD restriction enzyme analysis package (Lepais & Weir 2014) with different fragment size selection windows in order to obtain 20,000-30,000 regions according to Peterson *et al.* (2012). A size selection window of 450±44bp was selected based on the number of theoretical fragments (28,590) and size selection accuracy of the PippinPrep targeted size selection machine (Peterson *et al.* 2012).

Libraries were generated using a modified version of the Peterson *et al.* (2012) ddRAD protocol. Briefly, individual genomic DNA (~1 µg) was digested overnight with 10 units *EcoRI*-HF and 10 units *MspI* at 37°C. All digests were checked on a 0.8% agarose gel to ensure complete digestion. Digests were then cleaned using Sera-Mag SpeedBead Carboxylate-modified Microparticles (Thermo Scientific 2014) and quantified accurately using the Biotium ACCULBLUE High Sensitivity dsDNA quantification kit (Biotium 2013). Digested samples were standardised to 400ng and sorted into groups of 48. A ligation reaction was carried out where unique in-line barcodes and a common biotinylated adaptor were added to each fragment using T4 Ligase and buffer (Peterson *et al.* 2012). Ligated samples were then pooled into their sets of 48 for barcoding, cleaned using Sera-Mag SpeedBeads Carboxylate-modified Microparticles (Thermo Scientific 2014) to remove excess adaptors and reduce the volume to 50µl. These pools were quantified using a NanoDrop Spectrophotometer (Desjardins & Conklin 2010). No more than 5µg of each pool of 48 samples was loaded onto the PippinPrep targeted size selection machine (Sage Science Inc 2013) and a size range of 450bp ± 44bp was selected. A 2% Agarose gel cassette with Ethidium bromide and no overflow detection (Sage Science Inc 2013) was used to size select samples. Two elutions were taken along with a 0.1% Tween-20 rinse in order to maximise recovery. Eluate was cleaned using Streptavidin magnetic beads (Thermo Scientific 2014) to remove any fragments lacking a biotinylated adaptor. Illumina flow cell adaptors (P1) and one of 12 unique indexes (P2) were attached to each fragment via PCR using a Biorad C1000 thermal cycler: initial denaturation at 98°C for 30s, followed by 14 to 17 cycles of 95°C for 15s each, 66°C for 30s and 72°C for 45s and a final extension step at 72°C for 600s. Multiplexed PCR products were pooled and cleaned again using Sera-Mag SpeedBeads Carboxylate-modified Microparticles (Thermo Scientific 2014) to reduce the volume again to 50µl. Each pool of 48 was run on an Agilent 2100 Bioanalyzer (Agilent Technologies 2013) to ensure that size distribution was uniform between pools and to quantify for equimolar pooling prior to sequencing. The final multiplexed library was sent in a 20nmol concentration to the Australian Genome Research Facility (AGRF) for paired-end sequencing (101 bp) on the Illumina HiSeq2000 platform at an average read depth of ~1 million reads per individual.

Quality Control and SNP Filtering

The quality of raw sequences was screened using FastQC program (Andrews 2010) and reads with an average Q-score of <30 were discarded. To call individual genotypes, each library was processed through the *denovo_map.pl* pipeline in STACKs v1.20 (Catchen *et al.* 2011). This software de-multiplexes, quality checks, aligns and calls sequence variants across individuals and amongst populations. To avoid complications associated with mis-indexing, a combination of a unique 5bp barcode coupled with a 6bp index was used for each sample, in conjunction with the standard Illumina TruSeq indexes (Peterson *et al.* 2012). In order for a read to be retained, it needed to have all three barcodes completely intact, with no mismatching allowed. Any reads containing low quality or ambiguous barcodes were discarded. Following the extraction of individual trimmed reads, paired-end sequences were concatenated to form a single continuous read of 195bp, using custom bash scripts. Sequence variant calling in STACKs was conducted with default program parameters with the exception of mismatches when aligning loci within individuals (*ustacks* –m = 6), and further mismatches allowed when creating a reference catalogue of loci (*cstacks* –m = 3). Furthermore, a minimum sequencing depth (*populations* –m = 10) and a minor allele frequency (MAF) (*populations* –a = 0.02) were selected (Zenger *et al.* 2007), to create the final filtered genotype file. In order to minimise negative effects of missing data when calculating frequency-based genetic distance parameters (see Willing *et al.* 2012), only a single SNP was retained in each locus and only SNPs which were genotyped in >10 individuals and common to >2 populations. Additionally, all common autosomal loci deviating from Hardy-Weinberg equilibrium (HWE) in all populations were identified using the program Genetix (Belkhir *et al.* 1996) and removed if significantly ($P<0.01$) deviating from HWE. In order to identify contamination, all loci were searched against bacterial and viral databases (Johnson *et al.* 2008) and any matching regions were removed from the dataset. For frequency-based analyses, a sufficient level of individuals needed to be genotyped at a locus to make assumptions at a population level (Huang & Knowles 2014).

Sex-Linked Markers

Sex-linked loci were identified by comparing expected Mendelian patterns of loci in individuals of known sex (males=36, females=28). To ascertain if the loci matched expected patterns of autosomal loci, a Fisher's exact test (Altham 1969) was carried out with a correction for false discovery rate (FDR) of 10%. If the locus indicated sex-linked Mendelian inheritance (X or Y) in the test individuals, they were added to a short list. Meaning that to be short listed as X-linked, all males needed to appear homozygous (despite actually being hemizygous). To be shortlisted as Y-linked, all males needed to be homozygous and the locus was required to be missing in all females. As an additional test for Y-linked loci, deviation from HWE (in males) and linkage disequilibrium (LD) tests were conducted using Arlequin (Schneider *et al.* 2000) across all remaining individuals. Finally, shortlisted loci were evaluated against known marsupial sex chromosome sequence data (*M. eugenii* – Genbank accession

ABQO000000000.2 and *M. domestica* – Genbank accession GCF000002295.2). In order for a locus to be confidently classified as X- or Y-linked, it needed to adhere to all of the aforementioned tests. All short listed loci were removed from the autosomal dataset.

Population Genetic Diversity

To evaluate the genetic diversity within populations, standard diversity indices including average expected heterozygosity (He), average observed heterozygosity (Ho), allelic diversity and inbreeding coefficient (F_{IS}) were calculated through Genetix (Belkhir *et al.* 1996). Additionally, effective population size (Ne) was calculated with NeEstimator (Peel *et al.* 2004) using the linkage disequilibrium option (Ne_{LD}). To assess individual genome-wide diversity and inbreeding measures, multi-locus heterozygosity (MLH) and internal relatedness (IR) were calculated for all individuals using the R package *Rhh* (Alho *et al.* 2010).

Broad-scale Population Divergence

To illustrate the usefulness and consistency of this dataset, a number of different analyses were performed to assess broad-scale diversity and divergence. Genetic differences between populations and their significance were evaluated using Weir and Cockerham's unbiased F-statistics (Weir & Cockerham 1984), and hierarchical analysis of molecular variance (AMOVA) between populations, states, and regions (north, middle and south east coast) was calculated in Arlequin (Schneider *et al.* 2000). Broad-scale relationships between populations were visualised by constructing a neighbour-joining (NJ) genetic distance tree with Nei's standard distances (Nei *et al.* 1983). Genetic distances were calculated using Microsat2 (http://genetics.stanford.edu/hpgl/projects/microsat/) and tree reconstruction was performed using Mega6 (Tamura *et al.* 2013). Population assignments were confirmed using an assignment test carried out using GeneClass2 (Piry *et al.* 2004). A principal component analysis (PCA) using prior population clustering was conducted using the R package, *adegenet* (Jombart 2008) and was subsequently visualised through a DAPC scatterplot. The populations of Campbelltown and South Gippsland were excluded from the PCA as small sample size and lower genotyping rate were found to bias results.

Fine-scale Population Structure Resolution

The utility of genome-wide loci to unravel fine-scale genetic structuring was assessed across all individuals in the Queensland populations (St Bees Island, St Lawrence, Koala Coast and Ipswich) as well as in the large, isolated Port Macquarie population. Family groups were identified through the calculation of maximum likelihood (ML) estimates of relatedness and their relationships within the program ML-Relate (Kalinowski *et al.* 2006). Individual relationships amongst all individuals within the QLD populations and across all populations were then calculated and visualised using the NETVIEW pipeline v0.5.1 (Steinig *et al.* 2015) at kNN values between 5 and 35.

Outlier Loci Detection

To identify any candidate loci under selection in three populations with low genetic differentiation (F_{ST}<0.10) within QLD (Ipswich, Koala Coast and St Lawrence), outlier analyses were conducted using a frequency-based approach using Lositan (Antao *et al.* 2008). To identify outlier SNPs in Lositan, a total of 50,000 simulations were run at an FDR level of 0.1 at a 95% confidence interval (CI), with a "Neutral" mean F_{ST} being used and a "Forced mean F_{ST}" being fitted under an infinite allele model. Biological replicates were used in the form of two environmentally similar areas; Ipswich and Koala Coast. Only markers which were highly differentiated from the simulated mean and which were present in >2 pairwise tests were called as outliers.

Results

Sequencing and SNP discovery

A total of 317,573,718 paired-end sequence reads were obtained across all 171 unique individuals. After quality filtering through the initial modules of the STACKs pipeline, 6.68% of total reads were discarded due to low quality scores (Q-score <30) and ambiguous barcode sequencing. These reads were clustered into a catalogue of 1,088,361 RAD loci across all individuals which were used to confirm genotyping calls. The median number of reads per individual was 750,299 and ranged between 148,615 and 3,037,452. An average of 33,019 stacks or 'loci' were recovered from each individual at an average read depth of 17.3 reads per stack. Based on a minimum read depth of 10 and a MAF of >0.02, 13,998 polymorphic SNPs were retained across all individuals. Higher levels of missing data were observed between geographically distant populations, which has also been noted in more recent studies involving the ddRAD protocol (Andrews *et al.* 2014). This is largely attributed to a higher likelihood of mutations in enzyme restriction sites in more divergent populations, leading to disproportionate sampling between individuals (Andrews *et al.* 2014). To overcome this problem and to test the robustness of the dataset, various levels of missing data were run through each analysis to ensure minimisation of bias. A significant change in both heterozygosity and F_{IS} was observed when evaluating populations with read depths of below 10 at each locus. To rule out the effects of missing data, a subset of 500 of the most common SNPs were re-analysed for F_{IS}. Results of this re-testing did not reveal any significant differences in F_{IS} values ($P>0.05$). Inbreeding coefficients were high in all populations and ranged from 0.11 to 0.32 with Koala Coast displaying the highest F_{IS} value (0.32). Missing data skewed F_{ST} and F_{IS} results when <10 individuals were genotyped at a locus within a population and so an extra filtering step to ensure >10 individuals were genotyped at each locus, in at least two populations was carried out on this dataset. Although there were some differences in SNP profiles between populations, the datasets were still large enough with sufficiently high density so that this SNP incompleteness did not affect the outcome in this case (Huang & Knowles 2014). Of the loci recovered, 311 were putatively identified as sex linked based on genomic database matches and these were removed from the 'autosomal' dataset, with 95 loci (X chromosome = 58, Y chromosome = 37) adhering to the two most informative identification criteria (Mendelian patterns and BLAST matches to marsupial sex chromosomal regions). Following selection of a single SNP per locus, conformity to HWE testing across populations, minimum number of individuals genotyped in each population and screening for sequence contamination, a final set of 3,060 polymorphic autosomal SNPs were retained for further analysis (Supplementary Material 2.1).

Population Genetic Diversity

Average observed heterozygosity (Ho) ranged from 0.23 through to 0.29 and average expected heterozygosity (He) ranged from 0.26 to 0.35 (Table 2.1). Estimated effective population size (Ne_{LD}) across individual populations ranged from 2.7 (Campbelltown 95% CI = 2.4 - 3.2) to infinity, with

Campbelltown returning the smallest Ne_{LD}, likely due to small sample size and sub-sampling effect. Cape Otway and Port Macquarie returned Ne_{LD} = 46.7 [95%CI 40.8, 54.4] and 116.8 [95%CI 109.8, 124.6] respectively (Table 2.1). Individual MLH averaged over each population ranged from 0.18 to 0.29, standardised MLH (sMLH) being highest in Cape Otway (Table 2.1) and no private alleles were identified in the filtered dataset. Average internal relatedness (IR), another measure of population diversity (Alho *et al.* 2010), ranged from 0.20 to 0.42.

Table 2.1. Genetic diversity indices for each koala population/region sampled.

State	Location	n	Ho	He	Fis (P <0.01)	Av. MLH (±SD)	sMLH (±SD)	IR (±SD)	Ne_{LD} (95% CI)
QLD	St Bees Island	19	0.29	0.35	0.23	0.29 (±0.06)	1.05 (±0.24)	0.29 (±0.15)	Infinite (∞)
QLD	St Lawrence	19	0.26	0.3	0.2	0.24 (±0.04)	1.03 (±0.15)	0.21 (±0.11)	Infinite (∞)
QLD	Koala Coast	24	0.22	0.3	0.32	0.18 (±0.09)	0.79 (±0.39)	0.42 (±0.29)	Infinite (921.20–∞)
QLD	Ipswich	23	0.27	0.31	0.19	0.25 (±0.06)	0.98 (±0.21)	0.26 (±0.16)	Infinite (∞)
NSW	Port Macquarie	45	0.23	0.28	0.21	0.23 (±0.05)	0.94 (±0.20)	0.25 (±0.15)	116.8 (109.8–124.6)
NSW	Campbelltown	9	0.27	0.33	0.27	0.25 (±0.11)	0.97 (±0.41)	0.34 (±0.27)	2.7 (2.4–3.2)
VIC	South Gippsland	19	0.24	0.3	0.27	0.24 (±0.11)	1.01 (±0.48)	0.31 (±0.34)	Infinite (∞)
VIC	Cape Otway	13	0.24	0.25	0.11	0.24 (±0.04)	1.07 (±0.16)	0.20 (±0.11)	46.7 (40.8–54.4)

Broad-scale Population Divergence

Pairwise F_{ST} values between populations displayed varying levels of genetic differentiation, ranging from 0.28 between St Lawrence and South Gippsland, to 0.01 between Koala Coast and Ipswich - the two closest sampled populations (Table 2.2). Interestingly, despite being geographically further apart, Port Macquarie revealed less differentiation from the population in Ipswich, QLD (F_{ST} 0.11) than from Campbelltown, NSW (F_{ST} 0.13). Differentiation between mainland populations within QLD was low (F_{ST} 0.01 - 0.08) when compared to the two Victorian populations (F_{ST} 0.10). Assignment tests correctly assigned 100% of individuals to their source population, with only 15.85% of individuals being assigned to a second, geographically close population. Broad-scale genetic structuring using NETVIEW revealed three major genetic clusters across the sampled populations, with the QLD populations in one large group, Port Macquarie clustering out on its own, and Cape Otway clustering apart from Port Macquarie (Figure 2.2a). Principal component analysis through DAPC (Jombart 2008) indicated three genetic groups (Figure 2.3), again with Port Macquarie and Cape Otway forming their own defined clusters, similar to the NETVIEW clustering. South Gippsland and Campbelltown populations were excluded from these analyses due to low sample size and less complete genotyping. The NJ distance tree also indicated three broad genetic clusters across the sampled populations, however here the NSW populations grouped more closely with the QLD populations, than with the southern groups (Figure 2.4). To further assess this demarcation of genetic groups, a hierarchical AMOVA was carried out within state boundaries, as per current sub-species classifications and with clustering groups revealed in DAPC and NETVIEW analyses. Only 11.19% of variation could be accounted for between sub-species groups, with a larger portion of variation being observed among populations within groups (28.29%) and among individuals within populations (46.94%). These results are not in keeping with current sub-species classifications (Goldfuss 1817 in Iredale & Troughton 1934; Thomas 1923; Troughton 1935).

Table 2.2. F_{ST} values based on Weir and Cockerham's unbiased genetic distances (1984).

	St Lawrence	St Bees Island	Ipswich	Koala Coast	Port Macquarie	Campbelltown	South Gippsland
St Bees Island	0.1	+	+	+	+	+	+
Ipswich	0.06	0.15	+	+	+	+	+
Koala Coast	0.08	0.14	0.01	+	+	+	+
Port Macquarie	0.15	0.2	0.11	0.13	+	+	+
Campbelltown	0.19	0.21	0.15	0.17	0.13	+	+
South Gippsland	0.28	0.22	0.23	0.25	0.25	0.2	+
Cape Otway	0.24	0.21	0.23	0.24	0.21	0.21	0.1

+ denotes value significant to P <0.05.

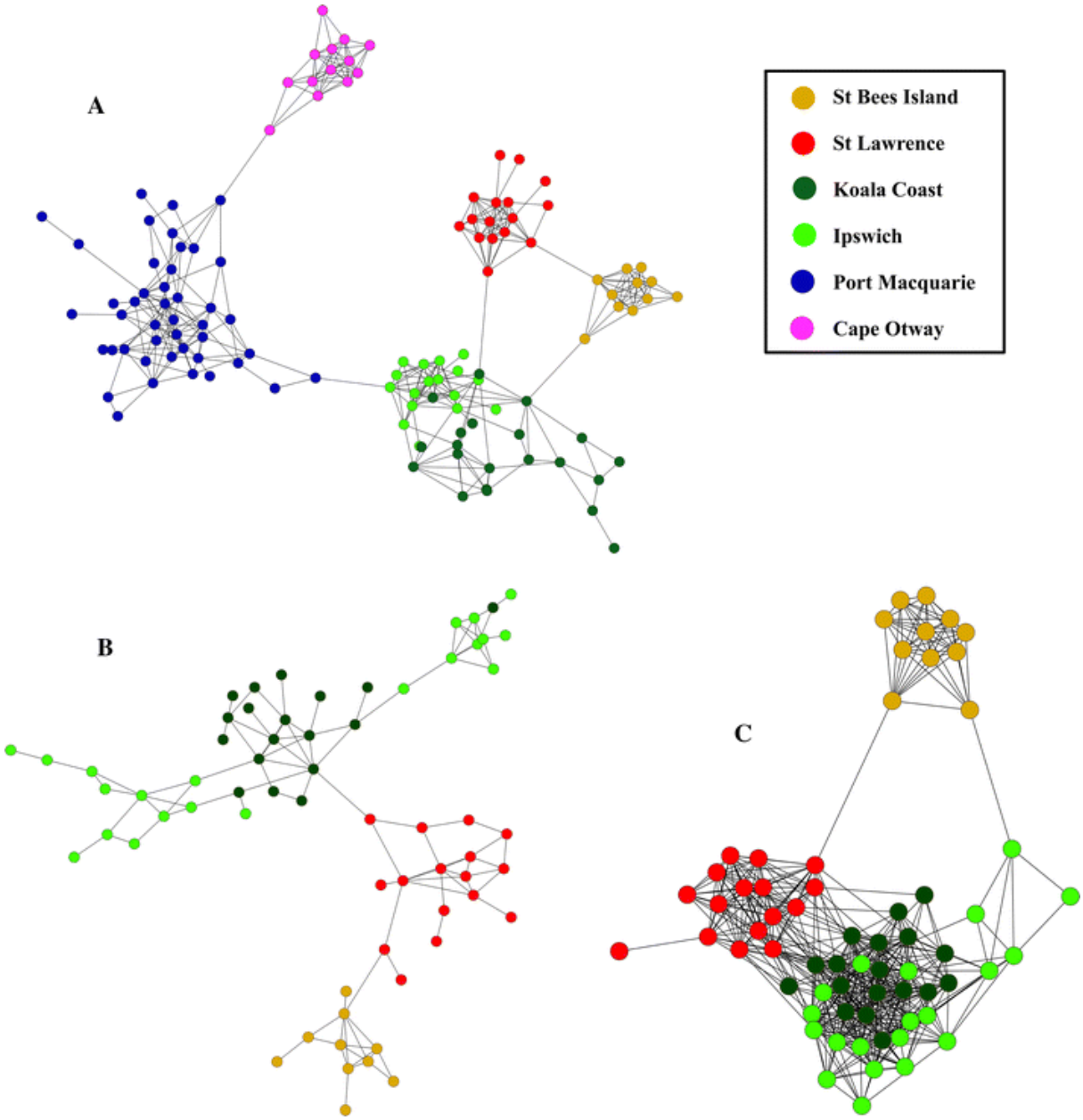

Figure 2.2. a) Population clustering of all populations using an isolation by state (IBS) matrix constructed using the NETVIEW v5.0 pipeline visualised at kNN=10. b) and c) Population clustering of all QLD populations using an isolation by state (IBS) matrix constructed using the NETVIEW v5.0 pipeline visualised at kNN=5 (b) and kNN=25 (c).

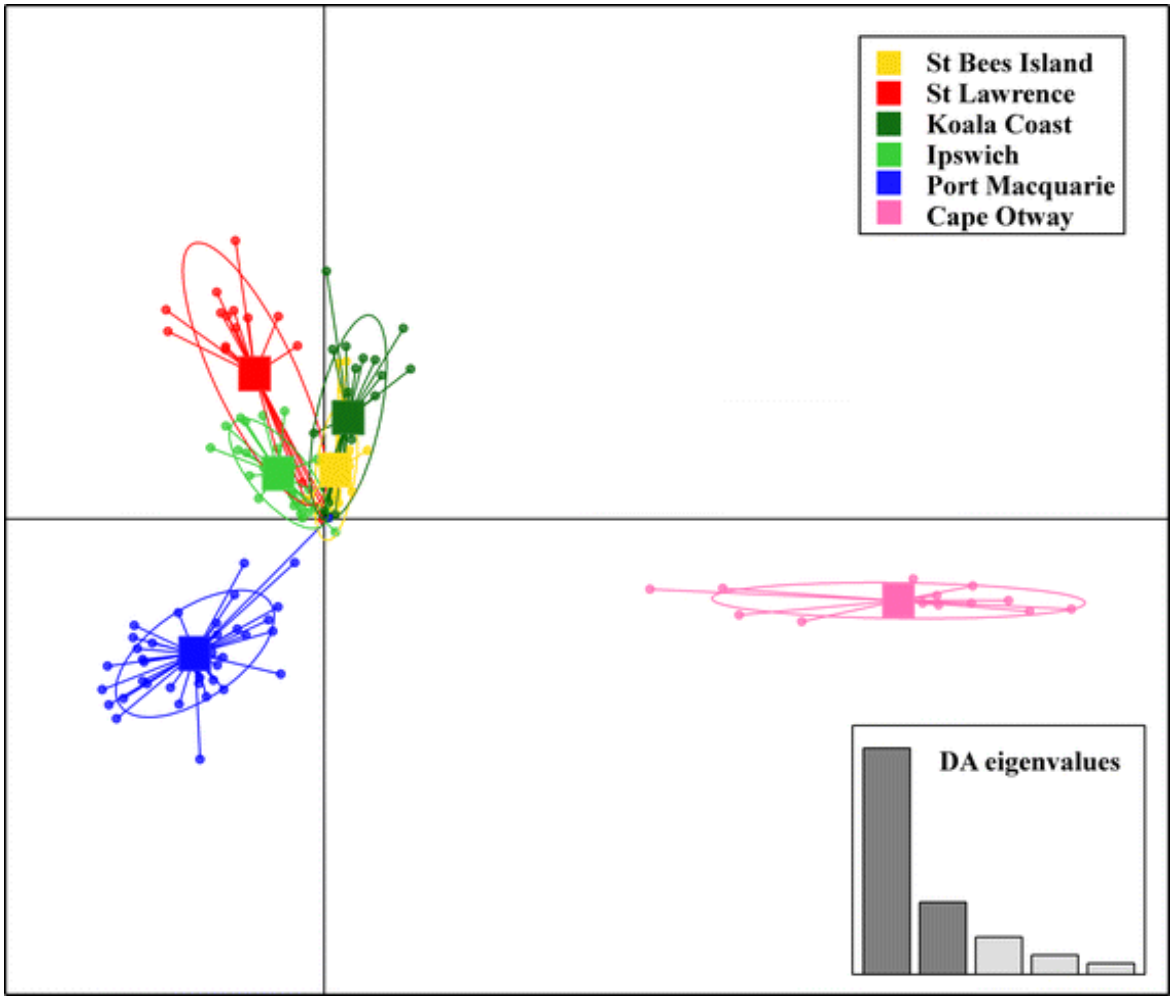

Figure 2.3. DAPC scatterplot created through the R package *adegenet* with prior population clustering.

Fine-scale Population Structure Resolution and Adaptive Variation

Within the QLD group of populations, NETVIEW clustering at kNN=5 (Figure 2.2b) was able to reveal fine-scale family structuring, showing small groups of 3-5 individuals clustering closely together. St Bees Island was the most distantly related to the mainland groups, with most genetic links being observed between St Lawrence and St Bees Island. A total of 31 half-sibling relationships (ML-relatedness >0.15) were identified in all QLD populations, along with 14 full-sibling and six parent-offspring pairings. Possible family groups were also identified within Ipswich, Koala Coast and St Lawrence, although at a NETVIEW clustering of kNN>10. These three populations appeared to be highly related (Figure 2.2b, c), despite St Lawrence being >600 km away from either Ipswich or Koala Coast. This relationship is also shown in low F_{ST} values (0.01 - 0.08) within this region. Due to its large sample size and distribution, the Port Macquarie population was also assessed for fine-scale structuring. In Port Macquarie (as within the QLD region), trio and small family groups were identified using NETVIEW analysis at kNN=5 and low level relatedness (0.01 – 0.10) was observed across most individuals sampled. A total of eight half-sib pairs, three full-sib pairs and one parent-offspring pair were identified using ML-relatedness measures. At a clustering level of kNN>10 in NETVIEW two sub-groupings begin to become apparent which mirror the sampling location of individuals (i.e. northern Port Macquarie and southern Port Macquarie region). The NETVIEW analyses of both QLD and Port Macquarie indicate strong localised sub-structuring within these regions, which is supported by inflated F_{IS} values (i.e. Wahlund effect).

Outlier loci were identified in three populations in QLD with low levels of genetic differentiation (F_{ST}<0.10) at an FDR of 0.1 using the frequency-based approach in Lositan (Antao *et al.* 2008). In total, 48 candidate directional outlier SNPs were identified as having a significantly higher F_{ST} than the simulated average between all QLD populations. Furthermore, 35 of these were common to at least two pairwise population tests indicating a high likelihood of these markers being biologically relevant.

Discussion

Population Diversity and Broad-scale Divergence

Refining transition zones and management boundaries is crucial to any future conservation efforts for the koala. Based on our results and the high level of diversity in morphology that has been observed in the koala (Houlden *et al.* 1999), a successful conservation plan for the koala must strike a balance between preserving a maximum level of diversity and avoiding potential complications from admixture, such as outbreeding depression (Frantz *et al.* 2006; Schwartz *et al.* 2007; Whisson *et al.* 2012).

The application of high throughput next generation sequencing of ddRAD loci allowed the development of the first large genome-wide SNP dataset in a diverse panel of koalas. From a practical perspective, the markers developed using this method will provide the tools for a much more comprehensive assessment of the genetic status of both wild and captive populations of the koala across much of its range. It will also allow for the development of more effective genetic management strategies, defining management boundaries for the species across all geographical cohorts and in captive breeding programmes.

The results outlined in this study have provided the first insights into genome-wide diversity in koala populations across their range. Overall diversity within populations was revealed to be equivalent to other vertebrate species, including canid, felid and ungulate species (Table 2.3). When compared to other vertebrate species genotyped using SNP markers and with a similar ecology and anthropogenic history, the populations sampled in this study show equivalent or greater values of both observed and expected heterozygosity (Table 2.3). Compared to large, constant populations such as wolves in North America (Gray *et al.* 2009) and feral pig populations in Europe (Goedbloed *et al.* 2012), the koala showed notably higher levels of diversity when assessed using genome-wide markers. Furthermore, multi-locus heterozygosity (MLH) was also revealed to be high in koalas in this study (MLH = 0.23). In contrast, an inbred population of harbor seals and a strain of outbred mice (Hoffman *et al.* 2014) demonstrated a lower level of MLH (0.06 and 0.16 respectively). Despite a general view that koalas have reduced genetic diversity across their range, based on limited studies using neutral markers (Lee *et al.* 2010; Melzer *et al.* 2000b; Phillips 2000), the results of this SNP study, which included samples from 5 of the sites used in previous studies (although not the same animals), indicate that in fact they may have equivalent genetic diversity to other stable outbred wild taxa (Table 2.3). It is worth noting that while species-wide diversity was generally high compared to the aforementioned taxa, there was considerable variation in levels of diversity within the studied koala populations. The Koala Coast population showed a reduced level of diversity when compared to the others sampled in this study, specifically showing lower heterozygosity and higher average IR across all individuals (Table 2.1). This population has at least one recorded historical bottleneck and has shown a similar trend in other studies (Lee *et al.* 2010). Heterozygosity was generally high in St Bees Island, in contrast to other island populations, such as Kangaroo Island, which have been reported to be highly inbred (Lee *et al.* 2012a).

Our results concur with another study that suggested St Bees Island may be one of the most genetically diverse of all island populations (Lee *et al.* 2012a). However, as no other studies have been conducted using genome-wide SNPs, it is difficult to make direct comparisons with other island groups. The lower levels of heterozygosity observed in South Gippsland are supported by extensive documentation of hunting and local extinction in Victorian populations in the early 20th century (Houlden *et al.* 1999; Wilmer *et al.* 1993). It would be useful to further sample animals in the Strzelecki region for comparison, as this population is said to have escaped much of the historical hunting and so may retain more historical diversity (Lee *et al.* 2012b).

While there is no known reproductive divide between groups of koala, this study indicates a significant level of genetic differentiation between geographic regions. All analysis performed indicated that northern and southern subgroups were genetically distant to one another, with Port Macquarie separating out on its own. Interestingly, Port Macquarie clustered more closely to some of the southern QLD populations than when compared with corresponding NSW groups despite geographic proximity to NSW groups. This trend has also been observed in MHC diversity studies across the species range (Lau *et al.* 2014). While it is clear that a general isolation by distance effect is being observed, further sub-sampling of populations within Northern NSW is needed to complete our understanding of the relationships between these groups. The AMOVA results showed very little differentiation between states overall (11.19%) and affirmed the lack of support for any sub-species assignment. The NJ tree (Figure 2.4) shows three genetic clusters, but also reveals that the NSW populations cluster more closely with populations in QLD, rather than their Victorian counterparts. This is supported by smaller F_{ST} values between these groups, and it appears that across Australia only two large genetic groups may be present, although a denser sampling strategy should be observed in future studies to confirm this. The largest variation between groups was observed between two of the most geographically distant mainland populations assessed in this study (St Lawrence, QLD and South Gippsland, VIC) and genetic distances between populations were generally high. However, F_{ST} values of 0.3 - 0.4 and a greater variation between sub-species groups would have been needed to provide support to any sub-species classification (Frankham *et al.* 2002), and this was not observed in this dataset. Genome-wide marker sets have been used to resolve sub-speciation and ecological management questions in a number of aquatic animal and plant species, including trout and rice (Feltus *et al.* 2004; Stephens *et al.* 2009). Similarly, the genome-wide SNPs developed in this study will help to resolve these issues in the koala, provided an additional and denser sampling strategy is employed to clarify potential 'transition zones'.

Effective population size can be an indicator of population health within a species. Given that this study only had access to a single temporal sample, a linkage disequilibrium method was used. Several population sample sizes were not sufficient to attain an accurate estimate of Ne_{LD}, with estimates for St Bees Island, St Lawrence, Ipswich and South Gippsland reporting infinite Ne values. The fact that most populations still returned an infinite Ne_{LD} indicates that the true values may be quite large, since if the

small number of individuals sampled were highly related, a small Ne_{LD} would have been expected. The two populations that returned robust values, Cape Otway (Ne_{LD} = 46.7 [95%CI 40.8, 54.4]) and Port Macquarie (Ne_{LD} = 116.8 [95%CI 109.8, 124.6]), both had the highest genotyping rate and Port Macquarie also had the largest sample size. Further sampling across multiple time periods and generations would be required for more accurate estimations, as it is possible that social sub-structuring may have skewed results in this case (Luikart *et al.* 2010). It is generally accepted that an Ne of 50 to 100 is sufficient for maintenance of short-term fitness (Shaffer, 1981), and an Ne of roughly 10% of the total census size allows a species to avoid an 'extinction spiral' (Frankel, 1981). An accurate national census size is difficult to attain in the koala as most estimates rely on public sightings, which can skew results, or small scale transect distance-sampling techniques, which are expensive and time-consuming so are rarely carried out on a large scale. Given that koalas have been regarded as having a hierarchical social system (White 1999; Mitchell 1990a, b) and general observed heterozygosity was high, this study concluded that the small Ne and the inflated F_{IS} values were due to social structuring and Wahlund effect (Sinnock 1975) rather than any excessive inbreeding within populations.

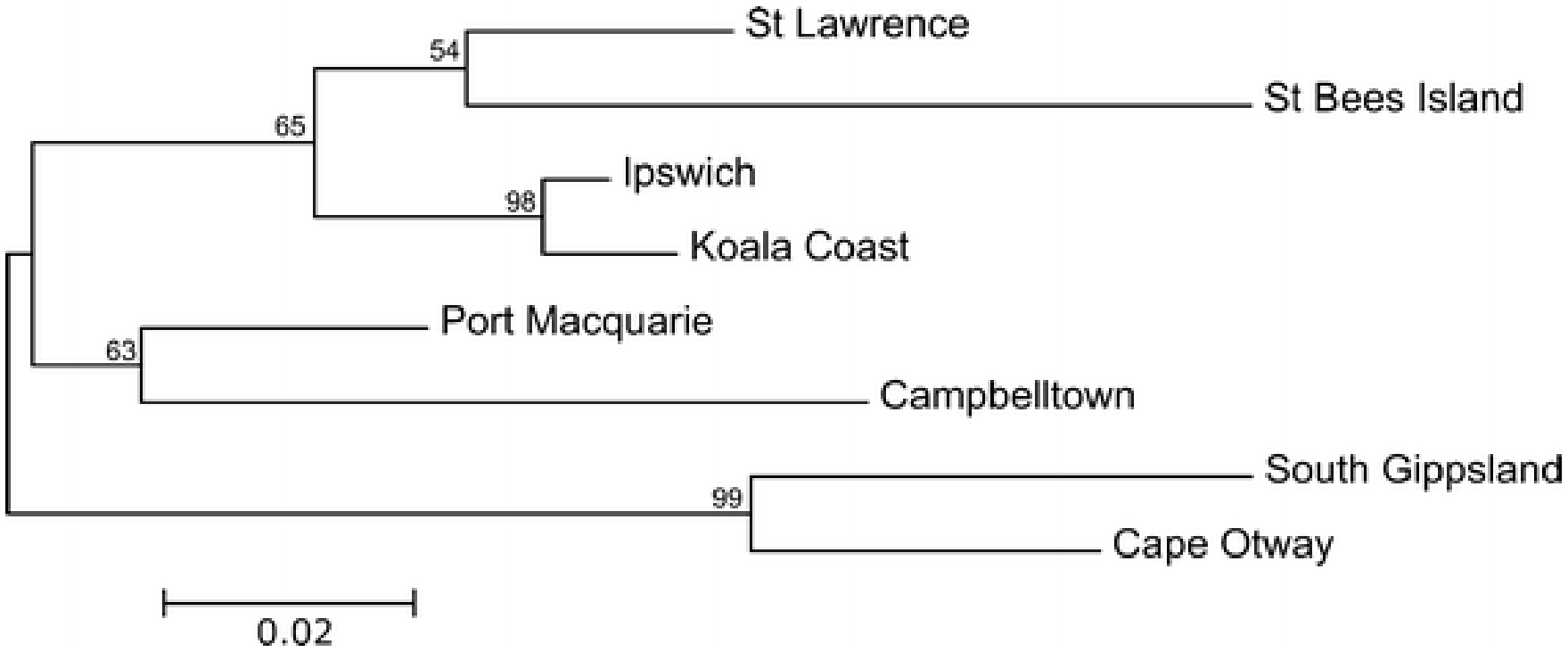

Figure 2.4. Evolutionary relationships between populations and regions, inferred using the Neighbor-Joining method with Nei's (1978) standard genetic distance. The optimal tree with the sum of branch length = 0.37 is shown.

Table 2.3. Heterozygosity values of various species based on SNP genotyping methods (listed until 2015).

Common name	Species name	He	Ho	References
Outbred or large, wild populations				
Deer	*Odocoileus spp.*	0.31	0.3	Haynes and Latch (2012)
Koala	*Phascolarctos cinereus*	0.29	0.27	This study
Feral Pig	*Sus scrofa domesticus*	0.28	0.27	Goedbloed *et al.* (2013)
Chinook Salmon	*Oncorhynchus tshawytscha*	0.26	0.25	Narum *et al.* (2008)
Coyote	*Canis latrans*	0.25	0.2	Kyle *et al.* (2006); Koblmuller *et al.* (2009)
Wolf (North American)	*Canis lupus*	0.24	0.22	Gray *et al.* (2009); Cronin *et al.* (2015)
Populations with a recent known bottleneck or domesticated species				
Domestic Cat (Russian Blue)	*Felis catus*	–	0.19	Kurushima *et al.* (2013)
Eurasian Beaver	*Castor fiber*	0.19	0.17	Senn *et al.* (2014)
Angus	*Bos taurus*	–	0.17	MacEachern *et al.* (2009)
Brown Bear	*Ursus arctos*	0.17	0.16	Cronin *et al.* (2014), Miller *et al.* (2012)
Wolf (Italian)	*Canis lupus*	0.17	0.15	Gray *et al.* (2009); Fabbri *et al.* (2007)
Arctic Ringed Seal	*Pusa hispida hispida*	0.14	0.13	Olsen *et al.* (2011)
Holstein	*Bos taurus*	–	0.12	MacEachern *et al.* (2009)
Polar Bear	*Ursus maritimus*	0.04	0.05	Cronin *et al.* (2014)
Black Bear	*Ursus americanus*	0.02	0.02	Cronin *et al.* (2014)

Fine-scale Population Structure Resolution

The ability of genome-wide markers to investigate fine-scale genetic structuring and relatedness (Cánovas *et al.* 2014; Consortium 2009; Kijas *et al.* 2009; Miller *et al.* 2012; Pollinger *et al.* 2011; Stephens *et al.* 2009) has been demonstrated successfully in this study, teasing apart relatedness and structuring in both the QLD region and Port Macquarie koalas. Within the QLD region, a historic link between the St Lawrence population and two south eastern QLD populations has been identified using genetic distance clustering (identity by state) through the NETVIEW pipeline (Steinig *et al.* 2015), indicating historical links given the extremely low probability of translocations between these areas. Lower levels of differentiation ($F_{ST} \leq 0.10$) and links defined within NETVIEW clustering (Figure 2.2b, c) are supported by historical records of translocations between central QLD and the introduced population of St Bees Island (Berck 1995). Our study also revealed there may have historically been active gene flow between koalas in the Ipswich region and the Koala Coast (Figure 2.2b, c), which are in close proximity but largely divided by significant barriers to dispersal, including large motorways and urbanised expanses. The genetic connectivity between these regions may be partly due to road mitigation measures implemented by the local governments to aid koala dispersal, such as raised wildlife crossings (Brisbane City Council 2014). It is also likely that haphazard translocations of rehabilitated animals by the public could have contributed to gene flow in this region. Further collaring and tracking studies are needed to confirm this.

A total of 95 sex-linked genetic markers were identified in this dataset. These markers could potentially be used in future studies to help investigate sex-biased dispersal and connectedness within and between closely linked populations. They may also allow for genetic tracking of paternity in the koala. Genetic analysis of sex-biased dispersal in the koala has until now largely been reliant on the use of *mtDNA* (Fowler *et al.* 2000; Taylor *et al.* 1997; Tsangaras *et al.* 2012; Wilmer *et al.* 1993), which can be inherently limiting due to its female biased inheritance pattern. A strong subset of sex-linked SNPs is an effective method to both confidently identify individuals and to test paternity (Heaton *et al.* 2002).

This study identified 48 putative outlier loci that were common to all QLD populations sampled indicating a high likelihood of local adaptation within these populations. A strong understanding of adaptive potential and the biology of a species are vital to a successful translocation and management plan. Koalas are already actively managed across their range in the face of growing human development, yet there is little genetic information available to guide this process. The identification of genetic sub-groups and management boundaries is critical for all genetically differentiated terrestrial species (Pollinger *et al.* 2010). While neutral markers such as microsatellites have been used extensively in wild populations to assess diversity within and between regions, more recent evidence suggests that a combination of adaptive and neutral markers such as those offered by a ddRAD dataset should be utilised when developing management plans in order for them to be most effective (Féral 2002; Fraser & Bernatchez 2001; Funk *et al.* 2012). Future studies are recommended to employ a more rigorous

sampling design (higher sample numbers) and couple this with environmental and phenotypic/morphological data in order to pinpoint regions of the koala genome under selection. Additionally, the utility of this method to identify markers potentially under selection could be used to inform future translocation planning for this species (Funk *et al.* 2012).

Usefulness of ddRADseq and limitations of the dataset

Our findings support the general findings that ddRAD has proven to be a cost effective (~$20 per sample) and efficient method for rapid discovery of large genome-wide datasets in a number of species (Peterson *et al.* 2012). Recent studies have indicated that the power of a marker set increases with a greater number of markers utilised (Miller *et al.* 2014) and SNPs have been demonstrated to be far more efficient and practical tool for population genetic studies than traditional neutral markers. In order to obtain the best quality SNP datasets, some forethought and planning needs to be conducted prior to library preparation. The *in silico* analysis performed prior to library preparation indicated that a size range of 350-450bp would recover between 20,000-35,000 regions in the koala genome. A range of 400±44bp was chosen as this was easily visible on an agarose gel image. The ddRAD protocol is dependent on high quality, genomic DNA and success relies on selecting enzyme combinations appropriate to genome content and structure. Thus, it is strongly recommended that an *in silico* digest is performed prior to library preparation and sequencing. The modified protocol used in this study maybe optimised to not only be applied to different species and taxa, but also gives flexibility in the number of regions of the genome recovered (Peterson *et al.* 2012). This allows for the fine-tuning of sequencing depth and the number of markers recovered. DNA preparation and standardisation between samples was also critical, as was evident when comparing sequencing runs 1 and 2, with the second run yielding significantly more reads per individual (84.9±0.03%). This was achieved through increasing consistency between samples at each step in the protocol and by decreasing the number of individuals pooled per sequencing run (~300 samples per lane). While the overall datasets can be improved by re-sequencing of libraries, this significantly increases cost and resource requirements.

Conclusions

The use of ddRAD sequencing and genotyping in conjunction with high throughput next-generation sequencing allows for a simple and effective method of genome-wide marker discovery. With careful planning and experimental design, this method has allowed for robust estimations of diversity and divergence in the koala; a species currently without an available reference genome (Andrews *et al.* 2014). This genomic resource is the first of its kind in the koala and will provide a basis for other genome-wide population studies in the future. The markers identified in this study have indicated that species-wide diversity in the koala is equivalent to, if not higher than other wild, outbred vertebrate species, but it is unclear how the koala compares to other marsupials, due to the lack of genome-wide research in the area. This finding is contradictory to a general view of the species as having low genetic diversity (Houlden *et al.* 1999; Tsangaras *et al.* 2012). This study has further resolved genetic groupings in the koala, showing three broad genomic clusters across Australia and a high level of variation between populations, indicating an isolation by distance model and rejection of a distinct sub-species classification, although a more comprehensive sampling strategy to pinpoint possible transition zones is needed to confirm this. The application of this method to a wider range of samples and populations across the species range will provide in-depth information that can inform conservation priorities, management and possible translocation plans for koalas across Australia.

Chapter 3: Broad-scale Genomic Differentiation and Taxonomic Classification

Genomic comparisons reveal biogeographic and anthropogenic impacts in the Koala (*Phascolarctos cinereus*); a dietary-specialist species distributed across heterogeneous environments.

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Research Objectives and Chapter Summary

Background/Gap in knowledge

The Australian koala is an iconic marsupial with highly specific dietary requirements distributed across heterogeneous environments, over a large geographic range. This specialized diet limits their potential habitat to regions able to support these eucalypt species.

There is currently limited information on population diversity and gene-flow at a species-wide scale, which also considers the potential impacts of local adaptation.

Aims

i) Examine the levels of genetic diversity in wild koala populations across the species range,

ii) Assess patterns of contemporary genetic structuring and connectivity between populations and bioregions,

iii) Provide insights into historical divergence among populations through phylogenetic reconstructions using genome-wide markers.

Significance/Conclusions

Genetic clustering analysis and phylogenetic reconstruction reveals a lack of support for current taxonomic classification of three koala sub-species, with only a single ESU supported

The Sydney Basin region is highlighted as a unique reservoir of genetic diversity, having higher diversity levels (i.e. Blue Mountains region; $AvHe^{corr}$=0.20, PL%=68.6).

Broad-scale population differentiation is primarily driven by an isolation-by-distance genetic structure model (49% of genetic variance), with clinal local adaptation corresponding to habitat bioregions.

Abstract

The Australian koala is an iconic marsupial with highly specific dietary requirements distributed across heterogeneous environments, over a large geographic range. The distribution and genetic structure of koala populations has been heavily influenced by human actions, specifically habitat modification, hunting and translocation of koalas. There is currently limited information on population diversity and gene-flow at a species-wide scale, which also considers the potential impacts of local adaptation. Using species-wide sampling across heterogeneous environments, and high-density genome-wide markers, I show that most koala populations display levels of diversity comparable to other outbred species, except for those populations impacted by population reductions. Genetic clustering analysis and phylogenetic reconstruction reveals a lack of support for current taxonomic classification of three koala sub-species, with only a single ESU supported. Furthermore, ~70% of genetic variance is accounted for at the individual level. The Sydney Basin region is highlighted as a unique reservoir of genetic diversity, having higher diversity levels (i.e. Blue Mountains region; $AvHe^{corr}$=0.20, PL%=68.6). Broad-scale population differentiation is primarily driven by an isolation-by-distance genetic structure model (49% of genetic variance), with clinal local adaptation corresponding to habitat bioregions. Signatures of selection were detected between bioregions, with no single region returning evidence of strong selection. The results of this study show that although the koala is widely considered to be a dietary-specialist species, this apparent specialisation does not limit the koala's ability to maintain gene-flow and adapt across divergent environments as long as the required food source is available.

Introduction

Specialist species evolve in stable environments to exploit available niches. However, specific adaptation to these environmental niches can make them more vulnerable to stochastic change than generalist species. Local persistence and dispersal rates of specialist species are strongly influenced by degree and type of ecological specialization (Li *et al.* 2014; Kierepka *et al.* 2016), and capacity to adapt to habitat change (Dennis *et al*. 2011; Hardy and Otto 2014). The level of ecological specialization can predict how well a species might survive in a recently modified landscape, and also how the species may adapt over time, which plays an important role in understanding species diversification (Dennis *et al*. 2011; Hardy and Otto 2014).

Specialist species often occupy smaller, more fragmented habitats and have smaller effective population sizes than their generalist counterparts (Horsák *et al*. 2012).Therefore, species with narrow ecological requirements are expected to be highly sensitive to further habitat loss and fragmentation (Franzèn *et al*. 2012; Kierepka *et al.* 2016). This leads to reduced gene flow and highly structured populations, which can increase the effects of random genetic drift, genetic bottlenecks, inbreeding and/or extinction events (Dennis *et al*. 2011; Li *et al*. 2014). Loss of genetic diversity and connectivity via these processes limits the evolutionary potential and can alter the evolutionary trajectory of the species.

Patterns of genetic differentiation vary considerably across specialist and generalist species (Packer *et al*. 2005). Specialisation in one dimension may lead to generalisation in another, or it may be context dependent, and specialisation may be restricted temporally or developmentally (reviewed by Li *et al*. 2014). Differences in selection pressures between populations due to ecological heterogeneity are potent drivers of evolutionary change. Understanding the genetic impacts of species-specific sensitivities to habitat changes is a crucial step towards formulating reliable predictions, which are valuable for understanding evolutionary processes, and informing conservation and management strategies (Murphy *et al.* 2011; Khimoun *et al.* 2014).

The koala is a marsupial with a specialised folivorous diet that can be found across much of the eastern coast of Australia (Figure 3.1). Koalas utilise up to 120 different species of tree across their distribution, but primary food tree species can be as few as two within a particular area (Melzer *et al*. 2000; Tucker *et al*. 2007). Furthermore, variability in chemical profiles even within a single eucalypt species can affect koala browsing preferences in different regions (Moore *et al*. 2005). This specialised diet limits their potential habitat to regions able to support these eucalypt species. Despite the koala's specialist dietary and habitat requirements, they are distributed across a vast range of environments and climatic zones from temperate to tropical regions. Corresponding climatic biomes range from sub-alpine forests in Victoria to sub-tropical forests in far north Queensland (Melzer *et al.* 2000; Penn *et al.* 2000; Phillips 2000). The koala's distribution is not continuous across this range and it occurs in a number of regions that are separated by large areas of cleared land or unsuitable habitat. As a consequence of

translocations, koalas now occur outside their natural range. These areas include many Victorian and Queensland Islands and South Australia (Melzer *et al*. 2000).

As is expected of an animal spanning a large range of varied habitats, the koala exhibits morphological differences (e.g. body size, pelage, and skull characteristics) between its northernmost and southernmost populations, with an intermediate phenotype in the middle of its range (Black *et al*. 2014; Briscoe *et al*. 2015). The phenotypic variation across the species range has led to koalas being classified into three separate subspecies; *P. c. adustus*, *P. c. cinereus* and *P. c. victor*. This classification was first described in the early 20th century, based on skull morphology and skins alone (Thomas 1923; Troughton 1935, 1941). There currently is no supporting genetic evidence for this taxonomic delineation. Genetic studies have attempted to understand taxonomic relationships using mitochondrial DNA (Houlden *et al.* 1999; Houlden *et al.* 1996; Neaves *et al.* 2016; Tsangaras *et al.* 2012), with results indicating a lack of support for the current sub-species classification. This outcome was also observed in a preliminary genome-wide SNP study, which again suggested that the current taxonomic classification should be re-addressed (Kjeldsen *et al.* 2016).

Determining appropriate species-wide management actions for the koala has been challenging due to limited information on broad-scale population connectivity and genetic structure across divergent landscapes. The current patterns of genetic diversity of koalas are likely to have been influenced by human activities, including clearing of habitat, hunting and translocations. These anthropogenic influences have been particularly strong in the southern states of Victoria and South Australia where the koala has a unique management history. A fur trade was established in the late 1800's and this, in combination with habitat destruction and wildfire, led to a dramatic decline in koala numbers (Menkhorst 2008). By the 1920's only a few remnant southern populations remained (Menkhorst 2008). However, concurrent with population declines on the mainland, koalas were introduced to islands outside their normal range in an attempt to salvage dwindling koala numbers, most notably French Island which was founded by as few as two or three individuals sourced from mainland Victoria (Menkhorst 2008; Houlden *et al*. 1996; Lewis 1934, 1954; Warneke 1978). The growth rate of the French Island population was so rapid that severe defoliation was observed within a short period of time (Martin & Handasyde 1999; Menkhorst 2008). From 1923 until the 21st century, koalas have been translocated from French Island to alleviate browse pressure. These translocations have created new populations, most notably Kangaroo Island which was reported as being established by 18 adult French Island animals (Masters *et al.* 2004). In almost a century of active management, koalas have been reintroduced to over 250 locations across Victoria (Menkhorst 2008). These management actions may have secured the future of koalas in Victoria, but at the cost of genetic diversity (Martin and Handasyde 1999; Menkhorst 2008). While population reductions may not have been as drastic in northern regions, within Queensland, several islands now support remnant or introduced koala populations (see Lee *et al.* 2012). During the 1930's, the St Bees Island koala population in central Queensland was founded from

as few as 12-17 individuals (Berck 1995), while the north Queensland Magnetic Island population was established from at least 18 individuals (Hrdina and Gordon 2004).

The conservation status of koalas varies across its distribution. Under Australian Federal law, the koala is classified as vulnerable in Queensland, New South Wales and Australian Capital Territory but is not listed in Victoria and South Australia. This dichotomy in conservation status is a reflection of the different overall population histories of koalas in these states and territories. Koala population declines have been observed across New South Wales and south east Queensland while some populations in Victoria and South Australia are so abundant that they are actively managed through translocation and fertility control to prevent defoliation of their preferred food trees and to prevent starvation (Menkhorst 2008; McAlpine *et al.* 2015; Whisson *et al*. 2016).

Although it has been widely accepted that substantial population size reductions and genetic swamping of remnant populations has had lasting effects on the genetic diversity of koalas in Australia (i.e. Menkhorst 2008), a recent preliminary study by Kjeldsen *et al.* (2016) reveals that population diversity is variable across the species geographic range. A number of other studies have also attempted to understand how the koala's ecological history has influenced genetic diversity. However, many of these were conducted on a local level, which limited their interpretation across the species distribution, or the investigators did not have access to genome-wide genetic markers, which is important for examining adaptive variation (Fowler *et al.* 2000; Houlden *et al.* 1999; Houlden *et al.* 1996; Lee *et al.* 2010; Lee *et al.* 2012; Neaves *et al.* 2016; Timms *et al.* 1993; Wilmer *et al.* 1993).

To date, there is limited information on connectivity and gene flow at a species-wide scale, while considering the potential impacts of local adaptation. Given the specialist nature of the koala distributed across divergent landscapes, it is important to understand how ecological or possible anthropogenic influences impact koala populations. Identifying levels of gene flow, genetic diversity and signatures of local adaptation will help inform at what geographical and/or ecological scale management should be implemented. In this study, using the most comprehensive genomic dataset to date, I aim to: 1) examine the levels of genetic diversity in wild koala populations across the species range, 2) assess patterns of contemporary genetic structuring and connectivity between populations and bioregions, and 3) provide insights into historical divergence among populations through phylogenetic reconstructions.

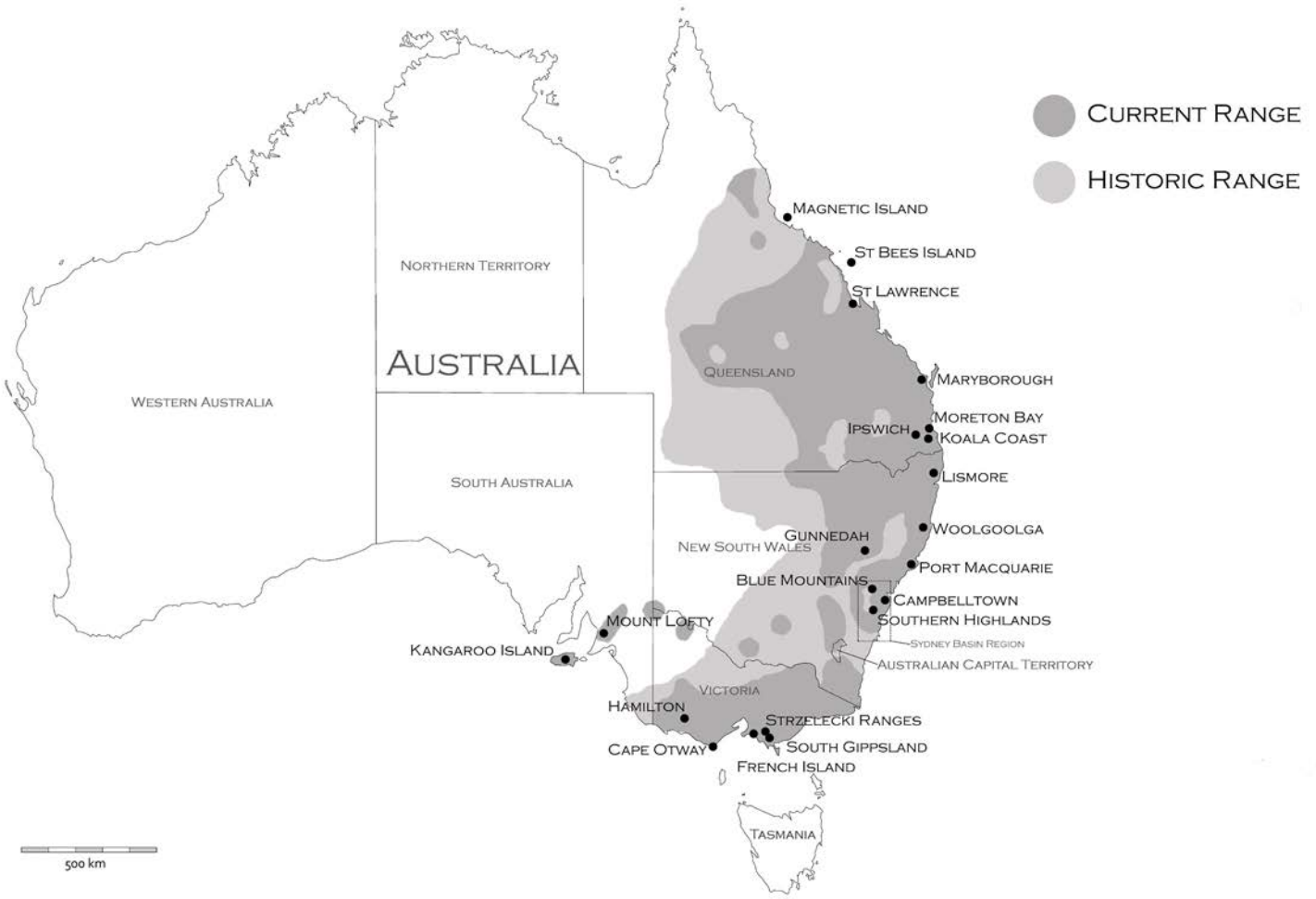


Figure 3.1. Distribution and current sampling range of *Phascolarctos cinereus* (currently and historically). Adapted from distribution map created by Strahan *et al.* (1995).

Methods

Sampling and DNA extraction

To ensure both natural and introduced koala populations were sampled across different bioregions and throughout the species distribution, a total of 21 representative regions (Figure 3.1), equating to 800 tissue or blood samples, were opportunistically collected from wild koala populations across eastern-Australia (see acknowledgments for further details). Tissue samples were preserved in 70% ethanol, while whole-blood samples were allowed to clot, before being stored at -20 °C. All DNA samples were extracted using a modified CTAB / Cholorform-Isoamyl method (Adamkewicz & Harasewych 1996) and further purified using a Sephadex G-50 approach (GE Healthcare Life Sciences 2000) to ensure no inhibitors were carried through to downstream genotyping.

Library Preparation and Sequencing

All samples were sequenced and genotyped using DArTseq™ genotyping technology at Diversity Arrays Technology, Canberra, Australia (Jaccoud *et al*. 2001, Kilian *et al*. 2012). Briefly, approximately 100ng (2µL) of each sample was digested with a combination of both a frequent and rare cutting restriction enzyme, *PtsI* and *SphI*, and unique barcode sequences simultaneously ligated onto the ends of each resulting fragment (Kilian *et al*. 2012). The *PstI*-specific adaptor incorporated an Illumina flowcell attachment region, coupled with a primer sequence, and unique barcode, with the reverse *SphI*-specific adaptor containing a second Illumina flowcell attachment sequence to facilitate bridge amplification (Lind *et al*. 2017). A minimum of 15% random technical replicates were included in all genotyping batches for downstream quality control. Each sample was individually amplified using proprietary barcode and adaptor sequences, with only fragments containing both *PstI* and *SphI* cut sites being amplified for sequencing, before being purified using a Qiagen PCR clean-up kit (Werle *et al*. 1994). Each sample was checked visually on an agarose gel to ensure complete digestion and conformity to a uniform range of fragment sizes. Samples which displayed incomplete digestion or a downshifted digestion pattern were removed from the library and not carried forward. Using approximately 10µL of each sample, batches of 288 samples were pooled for sequencing on a single flow-cell lane on the Illumina HiSeq2500 for 77 cycles.

Quality Control and Initial SNP Calling

DArTseq™ genotyping technology generates two independent marker types – Single Nucleotide Polymorphisms (SNPs) and Presence-Absence Variants (PAV, dominant loci) identified from restriction site-associated (RAD) fragments. SNPs were used for both population and phylogenetic analyses, while PAVs were only used in phylogenetic reconstructions. To ensure the highest quality loci were carried through to downstream analysis, the following sequence quality control and filtering measures were conducted. Raw sequence data in a fastq file format was obtained, and de-multiplexed according to individual barcodes. Each read was assessed for overall quality, and any reads containing base pair Q-scores <30 were removed. All reads were checked against existing sequences in the DArTdb

database (Sivasankaran *et al.* 1993) and also against viral and bacterial databases to assess contamination. If any contamination was identified, those reads were removed from the dataset.

SNP and PAV calling was performed using the *DArTsoft14* algorithm within the KDCompute pipeline developed by Diversity Arrays Technology (http://www.kddart.org/kdcompute.html). KDCompute SNP calling was carried out by creating clusters of identical reads at a population (or dataset) level, with three nucleotide mismatches allowed, then similar clusters were matched together to identify polymorphisms within sequence reads as described by Wenzl *et al.* (2004), and Lind *et al.* (2017). All monomorphic and tri-allelic loci were removed from the SNP dataset. In order for a SNP to be called by KDCompute, both homozygous and heterozygous forms were required to be present within the entire dataset. Following SNP identification, the following metrics were provided with the dataset; homozygote and heterozygote numbers, call rate, allele frequency, polymorphic information content (PIC), average PIC across all individuals, average SNP count, average read depth and reproducibility (based on random replicates). Further filtering was conducted using custom python scripts (Steinig 2016, https://github.com/esteinig/dartQC). SNPs with an average read depth of <10 (Nielsen *et al.* 2011) and MAF of <0.01 were removed from the dataset. If multiple SNPs were observed within a sequence read, only the SNP with the highest call rate across individuals and MAF were retained. Any sample / locus with a reproducibility of <95% and a call rate of <70% were removed from the dataset. To capture independent loci, SNPs in linkage disequilibrium (LD; r^2>0.2) were identified across all populations using PLINK (Purcell *et al.* 2007). From pairs of loci in LD, the SNP with the lower call rate and MAF value was removed. Each SNP was also assessed for deviation from Hardy-Weinberg equilibrium (HWE) using Arlequin v3.5 (Excoffier & Lischer 2010), and if a SNP significantly deviated from HWE (P<0.0001) in all populations, it was removed from the working dataset. Loci putatively identified as sex-linked were removed from the final SNP dataset according to Kjeldsen *et al*. (2016). PAV loci are scored as "0 or 1" and were extracted *in silico* from sequences obtained from genomic representations (Lind *et al*. 2017). PAV loci are based on a range of DNA variations in the restriction enzyme recognition sites. PAV loci were filtered manually with a MAF of 0.02 across the dataset and technical reproducibility of less than 100% according to Lal *et al*. (2016).

Population Specific Genetic Diversity

To evaluate the level of genetic diversity within and between populations / regions, standard diversity indices including average expected heterozygosity corrected for sample size (He^{corr}), average observed heterozygosity (Ho), inbreeding coefficient (F_{IS}), number of private alleles (Ap), and rare alleles (Ar; MAF <5%) were calculated through the Genalex v6.502 analysis package (Peakall & Smouse 2006). To assess individual genome-wide diversity and inbreeding measures, standardised multi-locus heterozygosity (sMLH), and internal relatedness (IR) were calculated for all individuals using the R package *Rhh* (Alho *et al.* 2010). Koalas are largely solitary animals, with structured social hierarchies, and display highly variable home range sizes (0.4–300 ha) (Davies *et al.* 2013). In order to identify

closely related individuals, relatedness metrics were assessed using a maximum likelihood approach in MLrelate (Kalinowski *et al*. 2006). Individuals returning high relatedness values (>0.25) were identified within each population to assist in the interpretation and account for potential bias in the data (Hansen *et al*. 1997).

Population Structure

Pairwise genetic divergence between populations was evaluated using Weir and Cockerham's unbiased F-statistics (F_{ST}) (Weir & Cockerham 1984) and Nei's unbiased genetic distance (Nei 1978) in Genalex v6.502 (Peakall & Smouse 2006). Genotypic relationships between individuals were visualised using the NetView R program (Neuditschko *et al*. 2014; Steinig *et al.* 2015) at multiple k-NN values (k-NN = 10-100). Optimisation of k-NN values was performed by plotting each k-NN value against the number of communities detected using a "Fast-greedy" clustering algorithm, following which k-NN values ranging from 40-60 were deemed the most appropriate based on this analysis.

Population structuring and proportion of genotypic admixture between populations and regions were also assessed using both a Maximum Likelihood approach in Admixture v1.3.0 (Alexander *et al*. 2009) and a Bayesian approach in Structure v 2.3.4 (Pritchard *et al*. 2010). To investigate models of gene flow, isolation by distance (IBD) mantel tests were conducted across the species distribution and for each geographic region in Genalex v6.502 (Peakall & Smouse 2006). In addition, to assess hierarchical levels of population structuring, an analysis of molecular variance (AMOVA) was calculated in Genalex v6.502 fitting geographic regions, populations and individuals as sources of variation (Peakall & Smouse 2006). The groupings for AMOVA evaluations were based on optimum network-based NetView clustering results (Neuditschko *et al*. 2012; Steinig *et al.* 2015; Supplementary Table 3.1), and on current sub-species classification and proposed population groupings in previous literature (Neaves *et al*. 2016).

Identification of signatures of selection

In order to identify loci that are under selection, outlier analyses were conducted using a Bayesian approach implemented within the program BayeScan 2.01 (Foll 2012). All South Australian and Victorian populations were excluded from these analyses, as population bottlenecks are known to affect outlier detection (Thornton & Jensen 2007). Analyses were conducted using 1:10 prior odds for a neutral model and all other parameters left as default (20 pilot runs of 5,000 iterations followed by 100,000 iterations with an additional burn-in of 50,000). Outliers were identified with a False Discovery Rate (FDR) of 0.001 and 0.01 using the Bayescan 2.01 function, *plot_R.r*. Both directional (alpha >0) and balancing or purifying (alpha ≤0) loci under selection were putatively identified. To help understand the impact of directional selection upon genetic relationships, the 1-proportion of shared allele distance matrix was calculated using the propShared function in *adegenet* (Jombart 2008) using both neutral and outlier loci. Individual relationships were then visualised using neighbour-joining (NJ) trees constructed

in MEGA6 (Tamura *et al*. 2013). Population pairwise F_{ST} values were also calculated independently for both neutral and directional outlier loci using Weir and Cockerham's (1984) unbiased approach based on 999 permutations within the Genalex v6.502 analysis package (Peakall & Smouse 2006). To investigate genetic signatures of selection amongst heterogeneous environments, populations were assigned to their specific bioregion according to Interim Biogeographic Regionalisation for Australia map (IBRA version 7, 2012; Table 3.1). Following the subtraction of neutral F_{ST} values from outlier F_{ST} values (to estimate the level of selective forces alone), the average within and between bioregion F_{ST} values were calculated.

Table 3.1. Diversity indices including: Populations Name and region, number of samples (n), Bioregion (based on IBRA version 7, 2012), corrected expected heterozygosity (He^{corr}), observed heterozygosity (Ho), percentage of polymorphic loci (%PL), inbreeding coefficient (F_{IS}), average F_{ST} between a single population and all others (AvF_{ST}), number of private alleles per population (#Ap), frequency of rare alleles (MAF<0.05) per population (Ar), standardised multilocus heterozygosity (sMLH), and internal relatedness (IR) *

	Population	n	Bioregion	He (corr) ±SE	Ho ±SE	%PL	Fis ±SE	avFst ±SD	#Ap	Ar ±SE	sMLH ±SD	IR±SD
1	Magnetic Island (MI)	20	BBN	0.14±0.00	0.14±0.00	47.8%	0.01±0.00	0.3±0.12	0	0.07±0.00	1.02±0.05	0.57±0.03
2	St Bees Island (SB)	21	CMC	0.14±0.00	0.14±0.00	53.8%	-0.03±0.00	0.32±0.13	0	0.08±0.00	1.07±0.36	0.55±0.14
3	St Lawrence (SL)	18	CMC	0.18±0.00	0.16±0.00	60.7%	0.07±0.00	0.21±0.11	0	0.11±0.00	1.1±0.16	0.55±0.10
4	Maryborough (M)	14	SEQ	0.15±0.00	0.14± 0.00	45.2%	0±0.00	0. 29±0.12	0	0.06±0.00	1.02±0.09	0.57±0.04
5	Moreton Bay (MB)	8	SEQ	**	**	**	**	**	**	**	**	**
6	Koala Coast (KC)	20	SEQ	0.17±0.00	0.16±0.00	59.6%	0.03±0.00	0.23±0.11	0	0.1±0.00	1.11±0.09	0.55±0.06
7	Ipswich (I)	22	SEQ	0.19±0.00	0.17±0.00	68.9%	0.07±0.00	0.21±0.12	0	0.13±0.00	1.2±0.13	0.50±0.07
8	Lismore (LI)	77	SEQ	0.17±0.00	0.15±0.00	74.5%	0.11±0.00	0.24±0.11	5	0.15±0.00	1.07 ±0.11	0.55±0.05
9	Woolgoolga (W)	9	NNC	**	**	**	**	**	**	**	**	**
10	Gunnedah (GD)	57	BBS	0.16±0.00	0.15±0.00	64.6%	0.06±0.00	0.26±0.08	7	0.11±0.00	1.03±0.15	0.49±0.10
11	Port Macquarie (PM)	85	NNC	0.18±0.00	0.17±0.00	80.9%	0.06±0.00	0.23±0.1	5	0.18±0.01	1.22±0.19	0.58±0.19
12	Blue Mountains (BM)	19	SYB	0.20±0.00	0.18±0.00	68.6%	0.1±0.00	0.15±0.06	0	0.18±0.01	0.99±0.35	0.63±0.11
13	Campbelltown (CT)	119	SYB	0.15±0.00	0.14±0.00	82.5%	0.03±0.00	0.32±0.08	2	0.12±0.00	1.1±0.27	0.53±0.11
14	Southern Highlands (SH)	25	SYB	0.18± 0.00	0.15±0.00	64.0%	0.08±0.00	0.22±0.07	0	0.09±0.00	1.06±0.15	0.56±0.05

15	South Gippsland (SG)	17	SCP	0.11± 0.00	0.1±0.00	37.7%	-0.01±0.00	0.29±0.12	0	0.04±0.00	0.73±0.06	0.70±0.09
16	Strzelecki (SZ)	19	SCP	0.11± 0.00	0.11±0.00	39.4%	-0.01±0.00	0.27±0.1	0	0.04±0.00	0.76±0.16	0.68±0.09
17	French Island (FI)	39	SCP	0.10±0.00	0.11±0.00	49.1%	0.09±0.00	0.36±0.08	0	0.09±0.00	0.61±0.24	0.80±0.15
18	Cape Otway (CO)	28	SCP	0.12±0.00	0.11±0.00	53.7%	0.08±0.00	0.23±0.1	0	0.07±0.00	0.67±0.34	0.75±0.04
19	Hamilton (H)	4	VIM	**	**	**	**	**	**	**	**	**
20	Mt Lofty (ML)	23	EYB	0.13±0.00	0.12±0.00	60.2%	0.01±0.01	0.42±0.09	0	0.04±0.00	0.59±0.12	0.76±0.12
21	Kangaroo Island (KI)	14	KAN	0.13±0.00	0.09±0.00	44.6%	0.19±0.01	0.3±0.12	0	0.04±0.00	0.59±0.33	0.83±0.11

*Note: metrics for populations with n < 10 should be considered with caution due to potential sub-sampling effects

** Metrics omitted due to low sample size

Phylogenomics

Phylogenetic relationships for all individuals across the species range were inferred using both SNP and PAV loci incorporating a Maximum likelihood (ML) approach in RAxML v8.2.0 (Stamatakis 2014). In addition, a Bayesian reconstruction method was implemented in MrBayes v3.2.6 (Ronquist *et al*. 2012) on PAV loci. Maximum likelihood phylogenies were reconstructed using a general time-reversible (GTR) model of nucleotide substitution (ASC_GTRGAMMA) for SNP data, and an optimized site-specific evolutionary rate model (ASC_BINCAT) for PAV loci. For both analyses, a gamma distribution rate for heterogeneity and a 'Lewis' method of ascertainment bias correction was applied (--asc-corr). Finally, a rapid bootstrap algorithm (--autoMRE) was implemented for each run to test the support for each of the nodes.

Bayesian phylogenetic analyses were carried out using PAV markers in MrBayes v3.2.6 (Ronquist *et al*. 2012) and a subset (n=399) of the most informative individuals (based on ML trees) to facilitate convergence of each run. Bayesian analyses consisted of two runs of 100,000,000 generations each and eight independent chains. Heated chains were set to Temp=0.10, with a 25% burn-in and a sampling frequency of 1,000. Dirichlet prior states were set to 48:52, which were calculated based on observed frequencies of absence ("0") and presence ("1"). Runs were completed if standard deviations between runs were below 0.05, and were independently assessed for convergence using Tracer v1.6 (Rambaut *et al*. 2014). In addition to consensus trees produced by both RAxML and MrBayes, maximum credibility consensus trees were constructed using TreeAnnotator v1.7.0 (Rambaut & Drummond 2013), with a burn-in of 10% and a posterior probability cut-off of 25%. Each consensus tree was then constructed using a neighbour-joining approach, and visualised and edited in FigTree v1.4.2 (Rambaut & Drummond 2012). All individuals were used to create an initial tree, however for clarity, a subset of only the most distinct individuals was used for construction of the final consensus tree for each statistical method.

Results

SNP Calling and Quality Control

A total of 15,004,234 sequence reads, corresponding to 19,187 polymorphic loci were obtained across 800 individuals from Diversity Arrays. Following genotype filtering, 35 individuals were removed from the dataset due to poor SNP coverage and 13,818 SNPs (72%) were removed from the dataset for violating filtering parameters, with low call rate being the primary factor. A total of 104 sex-linked markers (X chromosome = 86, Y chromosome = 18) were identified and removed from the working dataset, and the remaining dataset of 5,265 SNPs was then tested for conformity to HWE (3 SNPs removed) and LD (659 SNPs removed). A final set of 4,606 unique autosomal SNPs were retained for downstream analysis. A total of 22,022 PAV markers were initially identified across all individuals. Following filtering, a total of 6,102 PAV markers were retained for use in phylogenetic reconstructions.

Population Specific and Regional Genetic Diversity

Average observed heterozygosity (Ho) across populations ranged from 0.09 to 0.18, and average expected heterozygosity (He^{corr}) ranged from 0.10 to 0.20 (Table 3.1). Among populations, the Blue Mountains population displayed the highest heterozygosity values (He^{corr} = 0.20), while the French Island population showed the lowest levels (He^{corr} = 0.10). Percentage of polymorphic loci ranged from 37.7% (South Gippsland) to 82.5% (Campbelltown) (Table 3.1). F_{IS} values were generally close to zero, with an average of 0.04, and ranged from -0.03 (St Bees Island) to 0.19 (Kangaroo Island). Average sMLH was highest in Port Macquarie (1.21), whilst Kangaroo Island displayed the lowest level (0.58). Frequencies of rare alleles within populations ranged from 0.04 to 0.18 across the species range, with Port Macquarie and Blue Mountains populations having the highest levels (Table 3.1). When comparing regions based on phylogenetic clades (North versus South, see below), sMLH, %PL, Ar and Ap values were higher in populations residing in the northern region (Table 3.1 and Figure 3.1).

Broad-scale Population Structuring

Across the sampling range, two clear genetic clusters were identified through NetView R clusters at k-NN ≥40, with major regional clusters being observed at k-NN = 30 (Figure 3.2). The divide between the two overarching clusters was observed within the Blue Mountains population. Most Victorian and South Australian populations clustered closely together, with the exception of South Gippsland and Strzelecki sourced samples, which were distinct from the other southern populations, but indistinguishable from one another. The majority of individuals sampled from the same location clustered tightly to their pre-defined populations, indicating that designated populations were appropriate (Figure 3.2). Populations within a specific bioregion (IBRA version 7, 2012) also tended to cluster more closely together at all predefined k-NN clustering levels.

Both ML and Bayesian approaches (Admixture and Structure respectively) returned very similar results at their respective K values, and only the results for Admixture are presented here. For the Admixture

analysis, K >9 was optimal, with genetic admixture highest within the Blue Mountains and Gunnedah regions, which was consistent with the clustering patterns observed in NetView R at lower k-NN values. When K=2 was visualised, based on the two overarching NetView R clusters and phylogenetic trees (see below), admixture can be observed throughout NSW populations (with exception of the Lismore population), with higher levels of admixture observed within the Sydney region (Campbelltown and Southern Highland populations; Figure 3.3).

The results of the isolation by distance Mantel test revealed a moderate to strong correlation with genetic distance and geographical distance when evaluating all populations across the species range (R^2 =0.49; Figure 3.4a). When north and south regions were analysed separately, based on NetView R clustering, populations from both regions showed positive relationships between genetic and geographical distance, although less strong for the southern region (Figure 3.4b & 3.4c).

Pairwise F_{ST} values were highly variable ranging from 0.04 between the geographically close Blue Mountains and Southern Highlands in NSW, to 0.56 between St Bees Island and Mt Lofty which are close to the geographical ends of the species range (Table 3.2 and Figure 3.1). Populations with documented genetic bottlenecks displayed higher average pairwise F_{ST} values (i.e. French Island AvF_{ST} = 0.36±0.08, Mount Lofty AvF_{ST} = 0.42±0.09, Kangaroo Island AvF_{ST} = 0.3±0.12) compared to populations with more stable population histories (i.e. Blue Mountains AvF_{ST} = 0.15±0.06), indicating that these reductions may have skewed or inflated F_{ST} values (Pearse & Crandall 2004). Average pairwise F_{ST} across all populations was 0.27 (SD± 0.12), and overall average within the northern group (based on NetView R clustering) was lower than in the southern groups (0.17, SD ± 0.06 and 0.27, SD ± 0.12 respectively). Partitioning of genetic variance based on AMOVA tests revealed that among-individuals and within-individuals accounted for most of the genetic variation (~30% and ~40% respectively) independent of the groupings applied to populations. Among-population variance ranged from 14.3% to 20.4% between analyses, reflecting moderate population differences corresponding to other analyses. Among-groups as the source accounted for the least amount of genetic variance, with the largest variance of 13.6% recorded when populations were grouped together based on genetic similarities and optimum number of clusters (i.e. F_{ST} and NetView R data; Table 3.2 and Figure 3.2a), while the minimum of 8.4% was obtained when populations were grouped based on Neaves *et al*. (2016) proposed groups. Groups based on northern and southern regions (as defined by NetView R analysis; Figure 3.2b), accounted for 10.5% of the genetic variance, and while groups based on current sub-species classification described 13.0% of the variation (Supplementary Table 3.1).

Table 3.2. F_{ST} values between pair of populations with n>10, calculated using Weir and Cockerham's (1984) unbiased approach based on 999 permutations (bottom left matrix). Nei's (1978) unbiased genetic distance (top right matrix). All values reported were significant to P>0.01. *

		1	**2**	**3**	**4**	**6**	**7**	**8**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**20**	**21**
1	Magnetic Island		0.04	0.03	0.07	0.06	0.05	0.07	0.10	0.08	0.09	0.13	0.11	0.18	0.17	0.18	0.17	0.18	0.15
2	St Bees Island	0.17		0.03	0.07	0.07	0.05	0.07	0.10	0.07	0.09	0.13	0.11	0.18	0.18	0.18	0.17	0.18	0.15
3	St Lawrence	0.10	0.12		0.05	0.04	0.03	0.04	0.08	0.05	0.07	0.10	0.09	0.15	0.15	0.16	0.15	0.15	0.13
4	Maryborough	0.24	0.26	0.15		0.05	0.04	0.06	0.10	0.07	0.08	0.12	0.11	0.17	0.17	0.18	0.17	0.17	0.15
6	Koala Coast	0.19	0.22	0.12	0.17		0.02	0.03	0.08	0.05	0.07	0.11	0.09	0.16	0.15	0.16	0.15	0.16	0.13
7	Ipswich	0.16	0.19	0.09	0.14	0.06		0.02	0.06	0.04	0.05	0.09	0.08	0.14	0.14	0.15	0.14	0.14	0.12
8	Lismore	0.19	0.21	0.13	0.17	0.10	0.08		0.07	0.05	0.06	0.10	0.08	0.15	0.15	0.16	0.14	0.15	0.13
10	Gunnedah	0.28	0.30	0.21	0.25	0.22	0.20	0.20		0.05	0.04	0.09	0.07	0.13	0.13	0.14	0.13	0.14	0.12
11	Port Macquarie	0.21	0.21	0.14	0.19	0.15	0.12	0.14	0.15		0.04	0.08	0.06	0.13	0.13	0.14	0.13	0.13	0.11
12	Blue Mountains	0.23	0.26	0.16	0.21	0.16	0.13	0.16	0.12	0.11		0.04	0.02	0.07	0.07	0.08	0.07	0.09	0.06
13	Campbelltown	0.33	0.34	0.27	0.32	0.28	0.26	0.26	0.24	0.22	0.15		0.03	0.10	0.10	0.11	0.10	0.12	0.09
14	Southern Highlands	0.28	0.30	0.22	0.27	0.20	0.18	0.21	0.20	0.17	0.06	0.13		0.08	0.08	0.09	0.08	0.10	0.08
15	South Gippsland	0.43	0.46	0.37	0.42	0.34	0.32	0.34	0.35	0.32	0.20	0.33	0.21		0.00	0.01	0.01	0.05	0.02
16	Strzelecki	0.42	0.45	0.36	0.41	0.35	0.33	0.33	0.31	0.30	0.19	0.29	0.23	0.11		0.01	0.01	0.05	0.02
17	French Island	0.49	0.51	0.45	0.47	0.43	0.43	0.43	0.40	0.41	0.32	0.42	0.37	0.34	0.23		0.00	0.04	0.01
18	Cape Otway	0.38	0.40	0.32	0.36	0.30	0.29	0.32	0.29	0.29	0.15	0.28	0.19	0.08	0.09	0.22		0.04	0.01
20	Kangaroo Island	0.53	0.57	0.48	0.50	0.45	0.45	0.47	0.43	0.45	0.34	0.48	0.41	0.39	0.39	0.31	0.25		0.04
21	Mt Lofty	0.39	0.43	0.34	0.39	0.31	0.29	0.31	0.33	0.30	0.20	0.32	0.21	0.06	0.16	0.33	0.08	0.39	

*Note: metrics for populations with n <10 have been removed due to potential sub-sampling effects of low sample size.

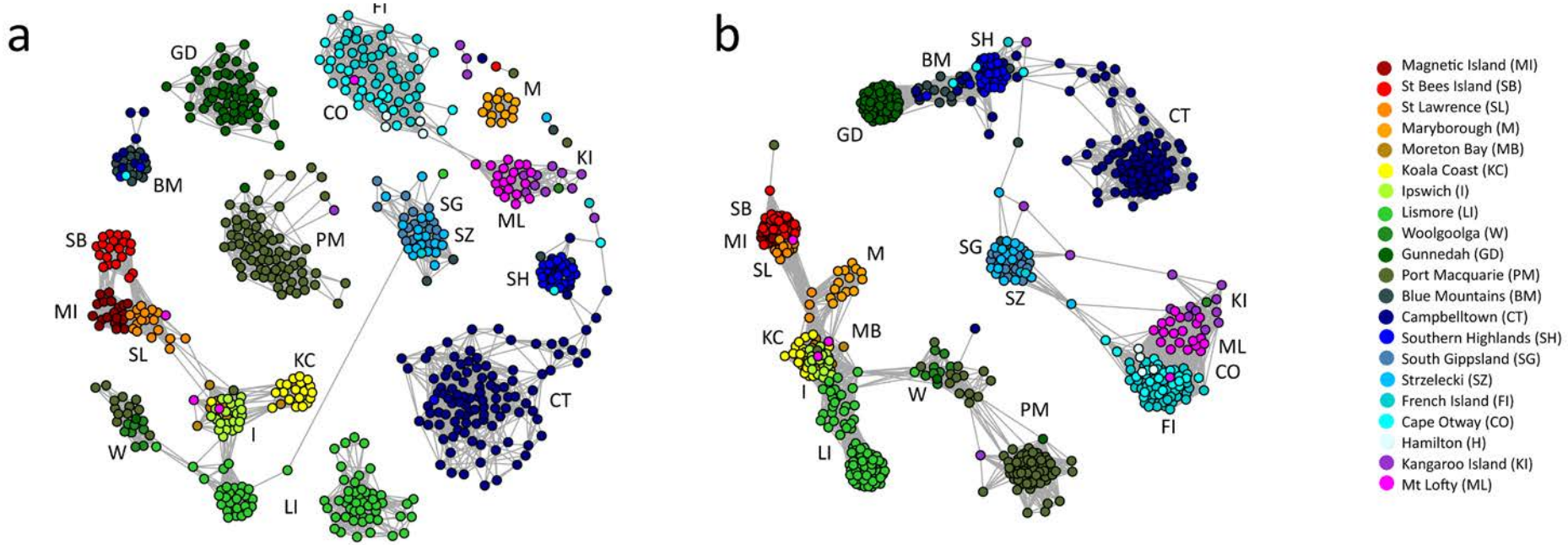


Figure 3.2. NetView R clusters at multiple k-NN values, a k-NN30, b k-NN60.

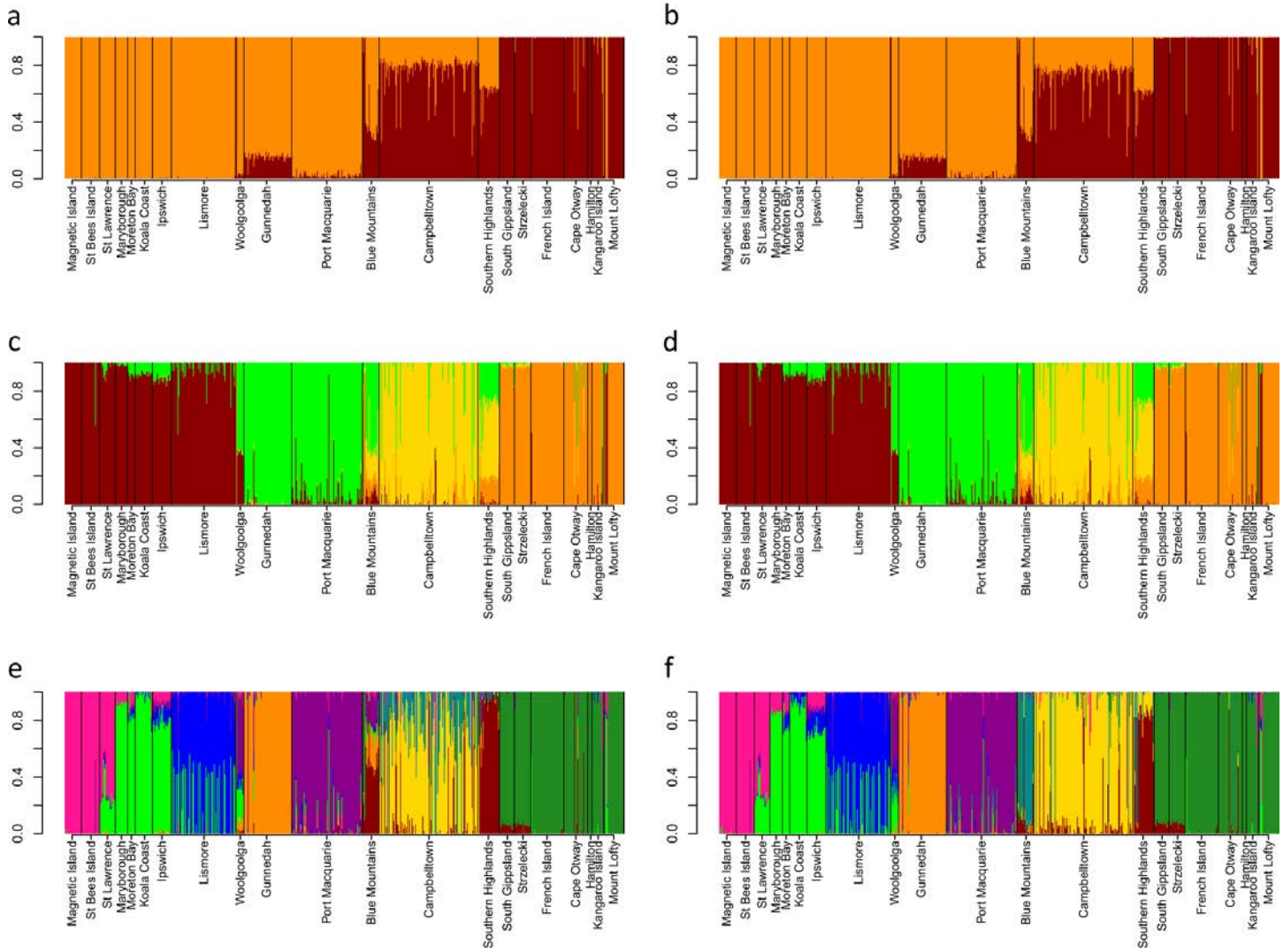
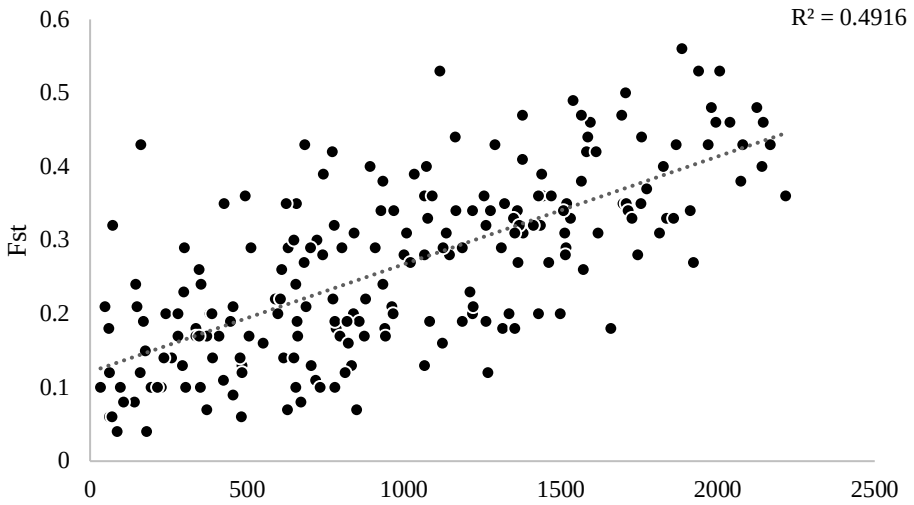
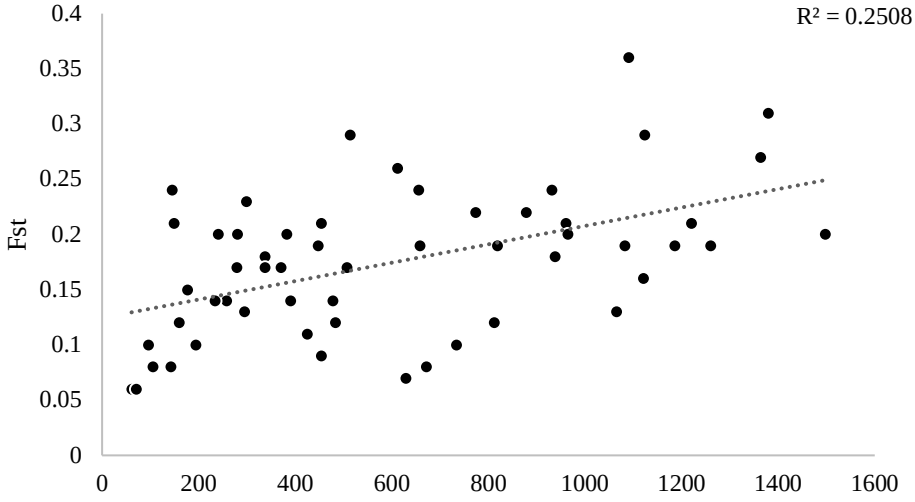


Figure 3.3. Proportion of genotypic admixture between regions calculated using a maximum likelihood approach in Admixture v1.3.0 (Alexander *et al.* 2009), and a Bayesian approach in Structure v2.3.4 (Pritchard *et al.* 2010); a) K = 2 (Admixture), b) K = 2 (Structure), c) K = 4 (Admixture), d) K = 4 (Structure), e) K = 9 (Admixture), f) K = 9 (Structure).

a)



b)



c)

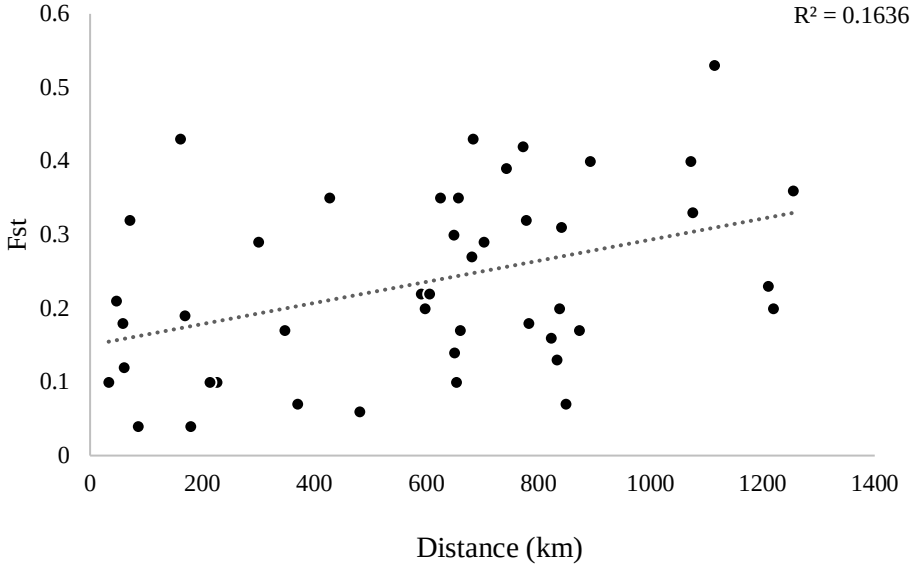


Figure 3.4. Mantel tests to investigate an isolation by distance model for gene flow between populations and regions, a) all populations, b) Northern populations, c) Southern populations.

Identification of signatures of selection

Weak to moderate signatures of selection were identified among populations investigated at both predefined FDR levels (FDR = 0.01 and 0.001). A total of 137 SNPs (100 directional and 37 purifying/balancing) were identified at FDR = 0.01, and 71 were identified at FDR = 0.001. Average F_{ST} across populations calculated using neutral loci was 0.18 (SD±0.06), with average F_{ST} for directional outliers being 0.37 (SD±0.17). When NJ trees were constructed based on 1-PSA genetic distances for each locus type, branch lengths were slightly longer and more uniform among individuals using neutral SNPs when compared to directional outlier SNPs (Figure 3.5). However, the directional SNPs displayed very similar clustering patterns across all populations, with no single population (or bioregion) showing signatures of extreme selection. When estimating the magnitude of population differentiation within and among bioregions due to selection alone, the average within bioregion F_{ST} was very low at 0.04 (SD±0.03) and average between bioregion F_{ST} was 0.20 (SD±0.11). Between-bioregion F_{ST} differences increased according to bioregion differences in a clinal pattern, whereby neighbouring bioregions displayed an intermediate average F_{ST} difference of 0.13 (SD±0.08), by comparison the greatest difference of 0.39 was observed between the most divergent bioregions (SYB verse BBN; Table 3.1).

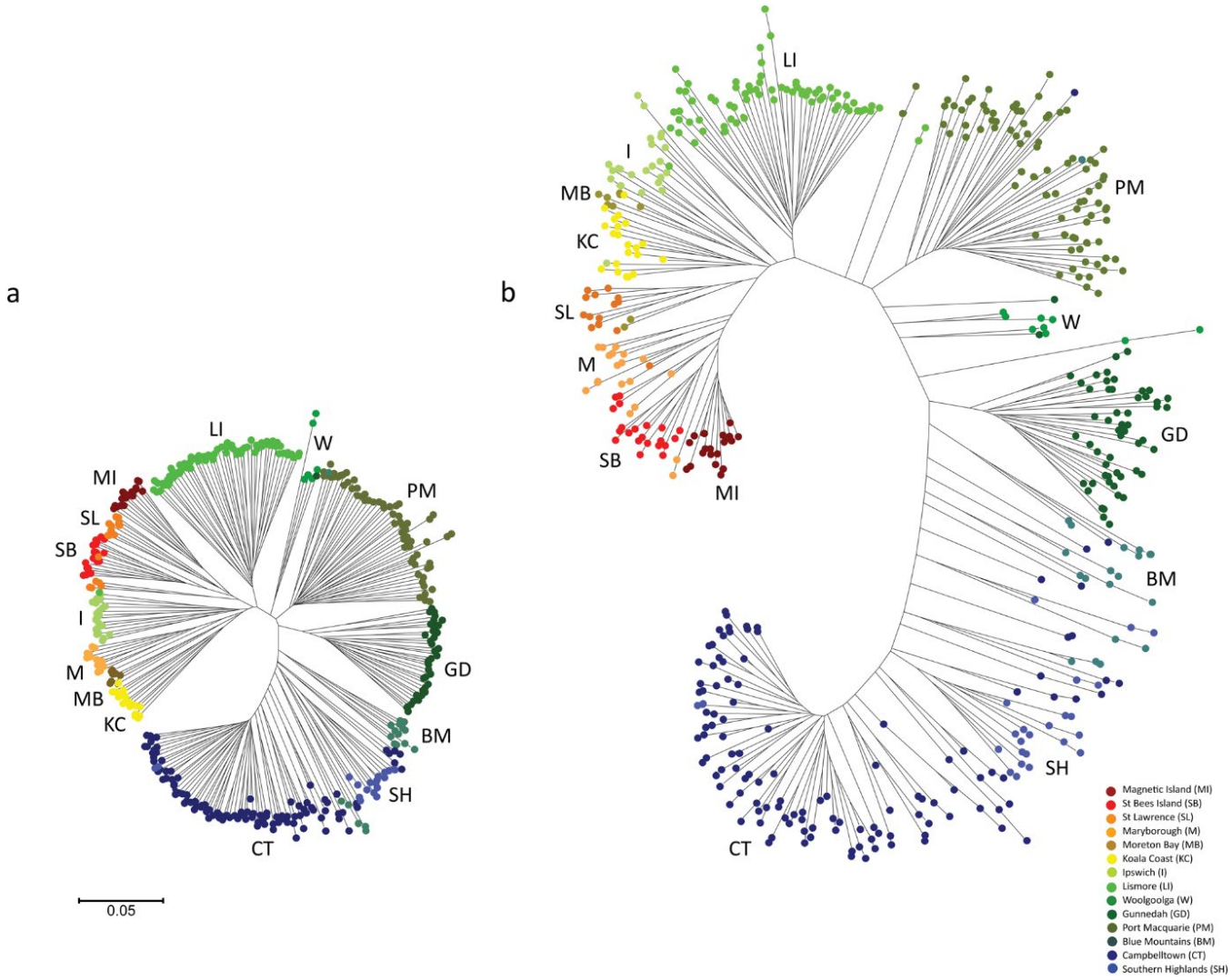


Figure 3.5. Genetic distance (1-Proportion of shared alleles) calculated based on a neutral and b putatively identified SNPs under selective pressures, trees constructed using a neighbour-joining approach in MEGA6 (Tamura *et al.* 2013)

Phylogenomics

Both maximum likelihood (ML) and Bayesian phylogenetic reconstruction, for both SNP and PAV markers revealed overall very similar topologies and node support with two major clades separating at the Blue Mountains/Campbelltown population in NSW (Figure 3.6; Supplementary Figure 3.2a, b). All populations north of the Blue Mountains (including the majority of Blue Mountains sourced individuals) clustered together in a single overarching northern group and all populations below Campbelltown (including the majority of Campbelltown individuals) clustered together in a southern clade (Figure 3.6; Supplementary Figure 3.2a, b). Individuals were generally placed within their assigned populations/regions with the exception individuals from Blue Mountains and Campbelltown, which had individuals in both the northern and southern clades. This was again consistent with NetView R clustering (Figure 3.2). Branch lengths were generally shorter among Victorian and South Australian populations, with the PAV trees placing all Victorian and South Australian samples as a subset clustering off the southern NSW populations (specifically the Southern Highlands group). Strong bootstrap support and posterior probability (>0.8) was observed for both major clades (north and south) in all reconstruction methods, with variable support at the intermediate nodes at a population level (0.42–1.0). A high degree of population mixing was observed within the southern clade, where individuals assigned to one population clustered with other populations (often a neighbouring population). Populations within Victoria and South Australia, with the exception of South Gippsland and Strzelecki, were intermixed in all phylogenetic tree reconstructions. South Gippsland and Strzelecki appear to be relatively divergent from the other southern koala populations, forming a distinct sub-clade apart from other southern populations in both phylogenetic constructions using PAV markers (Figure 3.6).

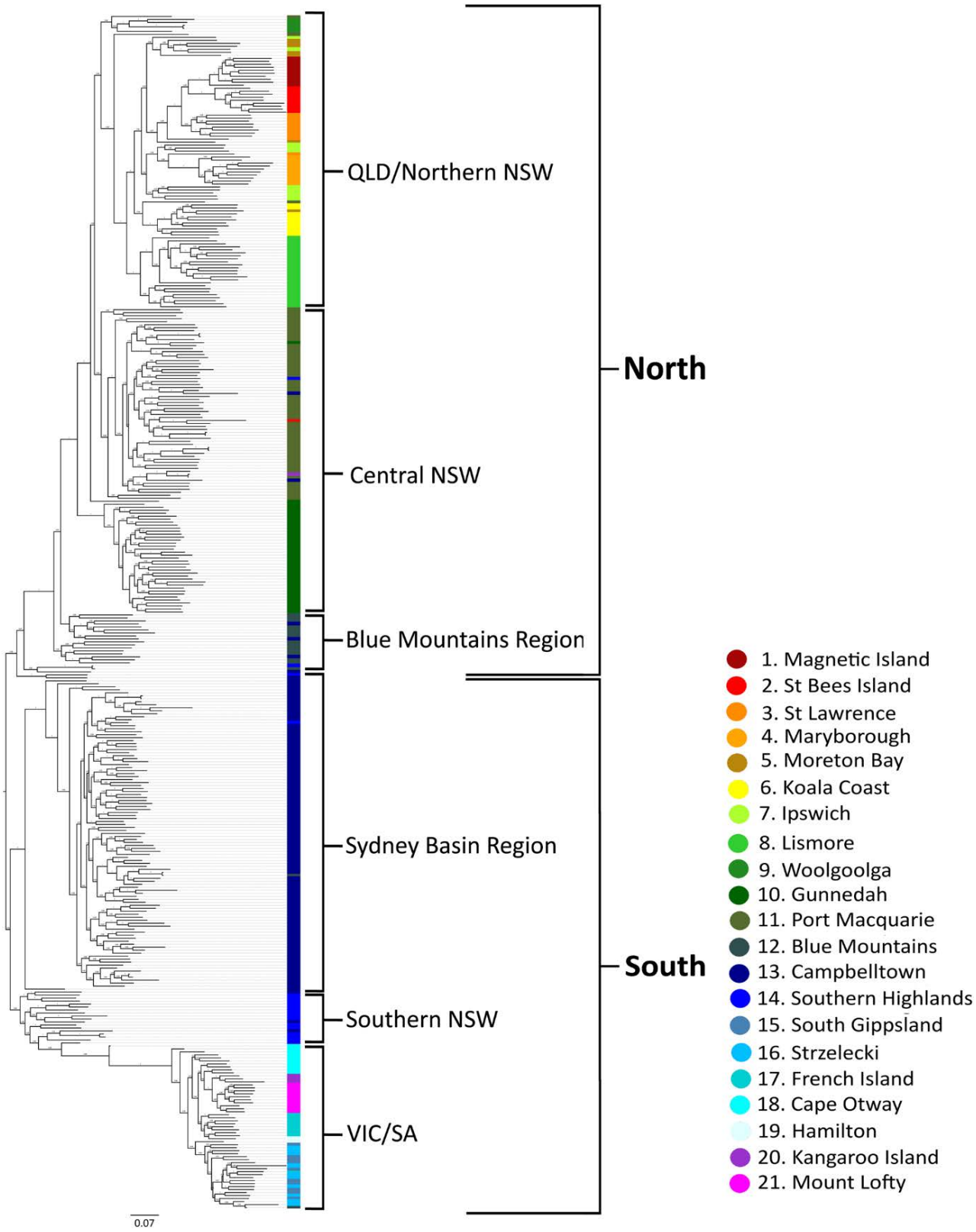


Figure 3.6. Phylogenetic reconstruction using a subset of 399 representative individuals. Tree constructed using a maximum likelihood approach based on PAV markers.

Discussion

Using a species-wide sampling strategy across heterogeneous environments, and high-density genome-wide markers, This study shows that remnant koala populations display comparable levels of diversity to that of other outbred species (Kjeldsen *et al*. 2016), and that their broad-scale population differentiation is primarily driven by an isolation-by-distance genetic structure model (49% of genetic variance) with clinal local adaptation. Detailed genetic structure patterns closely reflect population gene flow based on geographical locations, barriers to dispersal and documented translocations. Hierarchical genetic clustering analysis revealed two shallow overarching genetic groups present across Australia with genetic admixture, which is indicative of a weak historical genetic divide within the Sydney/Blue Mountains region. When assessing signatures of selection, the results of this study indicate that populations within bioregions are experiencing very similar selective pressures, while different selective forces are acting on bioregions in a clinal pattern across the koala's east-coast Australian distribution. Specialist species are expected to be more sensitive to selective pressures and stochastic change in the environment (Franzèn *et al.* 2010; Kierepka *et al.* 2016). However, our results indicate that the majority of koala populations are comparable in the levels of genetic diversity and sub-structuring (Table 3.2; Figure 3.2) to many other outbred species (Kjeldsen *et al.* 2016), except for those populations that have been impacted by population bottlenecks and/or translocations. The results suggest that although the koala is a widely considered to be a specialist folivore (Adams-Hosking *et al*. 2012; Hume 1982), this apparent specialisation does not limit the koala's ability to maintain gene flow and locally adapt across divergent environments. As long as specific diet requirements are met (Moore *et al*. 2005), they behave like a generalist species with no specialised environmental requirements to suit a specific bioregion. These genetic patterns can also be observed in the phylogenetic and AMOVA analysis presented here. These data indicate that at a taxonomic level, koalas belong to a single genetic group with the majority of genetic variation being between individuals, and as such does not support the three current sub-species classification.

Species-wide genetic divergence and signatures of selection

Previous koala phylogenetic studies have revealed between two and four genetic 'clades' across Australia using *mtDNA* sequence data (Houlden *et al.* 1999; Neaves *et al.* 2016). While *mtDNA* has been traditionally used for phylogenetic reconstruction across many species, it may not be ideal for resolving phylogenetic relationships in koala. The reported low level of koala *mtDNA* gene diversity (Houlden *et al.* 1999; Neaves *et al.* 2016) and absence of a suitable molecular clock (Neaves *et al*. 2016) limits its use in generating highly informative phylogenetic data with robust clade support. Genome-wide markers provide an added level of insight into the genomic structure and phylogenetic history of koala populations by sampling both adaptive and neutral regions of the genome (Kirk & Freeland 2011). Both phylogenetic analysis (Figure 3.6), and hierarchical clustering methods (Figures 3.2 & 3.3) utilised in this study reveal two historical shallow genetic groupings or 'clades' across Australia. The two

genetic groups have strong support of separation within the Sydney Basin, splitting the Blue Mountains and Campbelltown populations (Figure 3.1) between the northern and southern clades in all phylogenetic reconstructions. Despite this clear separation at one point in the koala's evolutionary history, admixture is also present within this region, indicating that even though historical barriers to gene flow have been present for a period of time, gene flow between northern and southern clades is currently occurring (Figure 3.3).

While difficult to confirm the specific barrier, based on the location of this genetic divide, it is possible that the 'Hunter Valley rift' contributed to this divergence. In studies on other species, including ancient assassin spiders (Rix & Harvey 2012), garden skinks (Chapple *et al*. 2011), giant burrowing frogs (Penman *et al.* 2005), common froglets (Symula *et al.* 2008), eastern yellow robins (Pavlova *et al.* 2013) and brush-tailed rock wallabies (Hazlitt *et al.* 2014), the Hunter Valley rift has been implicated in driving the speciation of several other species groups (during the mid-late Miocene era). Given the geological history within this region involving dramatic changes in landscape and vegetation structure (Byrne 2008; Dubey *et al*. 2010), it is possible for this historical barrier to have previously restricted movement of koalas. Furthermore, habitat type and terrain could also have played a role in koala divergence, with the Great Dividing Range (GDR) falling within the region of admixture observed in this study. Significant climatic variation during the mid-Pleistocene (Byrne 2008), causing shifts from warm and wet conditions, to cool and dry conditions, leading to habitat expansions and contractions (Dubey *et al*. 2010), may also have contributed to koala divergence. Interestingly, no significant divergence was observed when *mtDNA* markers were used for phylogenetic reconstruction (Neaves *et al*. 2016). The discrepancy between reconstructions may be a result of a number of factors, not the least of which being that two different genetic marker types, with different mutation rates, were utilised between these studies. Despite this, it is evident based upon the presence of clear genetic admixture (Figure 3.3), that the historical barrier is no longer significantly affecting gene flow in present day populations.

The current study strengthens support for rejection of the current sub-species classification (Houlden *et al.* 1999; Kjeldsen *et al*. 2016), which classifies koalas into three distinct sub-species (Thomas 1923; Troughton 1935, 1941), based largely on state legislative borders and morphological differences. There was no indication, in any of our analyses that three distinct evolutionary significant units (ESUs) are present based on previous classifications. Hierarchical analysis of molecular variances (AMOVA) indicated that among and within individual sources of variation accounted for most of the genetic variance (approx. 30% and 40% respectively; Supplementary Table 3.1). This indicates that most of the genetic variation and evolutionary potential within the species is observed at the individual level rather than in geographical regions or populations. Among group results produced the smallest proportion of genetic variance (8.4-13.7%) whereby all proposed groupings including current sub-species

classifications and others based on phylogenetic relationships (e.g. Neaves *et al*. 2016 and Figure 3.6) did not meet the thresholds to warrant distinct ESUs (i.e. sub-species; Fraser and Bernatchez, 2001).

There is no doubt that conservation of evolutionary processes and ecological viability of koalas is of fundamental importance. Based on adaptive divergence and population connectivity data, it is proposed that koalas should be classified under a single ESU. Firstly, based on koala distribution data (Figure 3.1.), there is no obvious geographic delineation of koalas into distinct groups with significant historical isolation. Secondly, contemporary reproductive isolation is not observed, with moderate gene flow observed between proximal populations throughout their distribution (Table 3.2). Mantel test results indicate that an IBD population structuring effect is evident across the species range and explains a large proportion of the genetic variance observed (R^2=49.2%, p <0.001). Although a historical North / South separation is observed within the Sydney Basin (Figure 3.6), the divergence between the two clades is small and contemporary admixture is high between the regions (Figure 3.3). Finally, koala morphological traits and genetic signatures of selection follow a clinal pattern across an environmental gradient rather than distinct groupings (Martin & Handasyde 1999; Table 3.1).

The clinal phenotypic variation observed in the koala across its distribution is not surprising, based on the large species range (from wet tropics in Northern Australia through to temperate climates in the Southern Australia) (Briscoe *et al*. 2015). Phenotypic variation has been observed in a number of other species across environmental gradients, including red deer (*Cervus elaphus*) (Post *et al.* 1997), *Bicyclus* butterflies (*Bicyclus sp*.) (Brakefield & Reitsma 1991), red squirrels (*Sciurus vulgaris*) (Réale *et al.* 2003), and even humans (*Homo sapiens*) (Campbell & Tishkoff 2010; Manica *et al.* 2007). Much of this variation is attributed to adaptation to different climates and habitats (Manica *et al.* 2007; Briscoe *et al*. 2015). Variable ecological pressures can result in different selective pressures across different bioregions (Gienapp *et al*. 2008). Overall climate and habitat type vary significantly from the northernmost regions of Australia, through to the southern regions, with large differences in temperature ranges, rainfall, soil types and vegetation structure (Hughes 2003). Interestingly, despite this variation in climate and habitat across the koala's range, no strong signatures of selection were identified in any specific bioregions or population (Figure 3.5). This suggests that the koala is capable of inhabiting and adapting to a broad variety of environmental conditions as long as suitable dietary components are available.

Population genetic diversity and sub-structuring

Ecological history appears to have a direct effect on contemporary genetic diversity levels, with all southern populations (populations sampled from Victoria and South Australia) displaying clear reductions of diversity (Table 3.1). Historic records of hunting and subsequent reintroductions (Menkhorst 2008) are likely to have led to the low diversity levels seen in this region. Hunting in the early 20th century decimated many Victorian mainland populations (estimated <1,000 animals left by

1930) and led to the complete extinction of South Australian populations (Menkhorst 2008). This is reflected in the lack of distinct population groupings seen in most mainland Victorian populations (Figure 3.6). Some animals within the Strzelecki and South Gippsland regions are said to have escaped hunting (Menkhorst 2008), and the current study indicates that these populations are distinct from the rest of the Southern populations (Figure 3.2 & 3.5), although overall diversity was still relatively low (He^{corr}=0.11 and 0.11 respectively). Animals that persisted in this region may have carried remnant diversity, and genotypes which were lost elsewhere in southern regions as a result of widespread hunting may have been retained in these populations.

Interestingly, population reductions do not appear to have adversely effected the koala's ability to thrive in the short term, as population numbers are increasing in several areas (e.g. Kangaroo Island and French Island). Genetic relationship patterns clearly show that most mainland Victorian koalas are very similar to those from French island (Figures 3.2 & 3.3), but the high F_{ST} values in this region are likely inflated due to repeated genetic bottlenecks and random genetic drift effects (Pearse & Crandall 2004).Variation in vegetation structure across the east coast of Australia may also affect abundance of animals across the species range (Davies *et al.* 2013; Dudaniec *et al.* 2013), although overall abundance within a region appears to be a poor indicator of genetic variation in this case.

Of the regions sampled in this study, groups of populations that were sampled from areas surrounding protected habitats (e.g. the Blue Mountains region) appeared to have higher levels of admixture, and generally higher diversity values than those surrounding suburban areas (Figure 3.3; Table 3.1). The higher levels of diversity in regions surrounding protected habitat may have been maintained by the increased genetic connectivity between these populations (Figure 3.2 & 3.3), reducing the effects of genetic drift, which can lead to random loss of alleles in smaller, isolated populations (Allendorf 1986). The dietary specialisation of koalas also restricts them to areas that can support their primary food tree species, and fragmentation of this habitat by either changes in climate, or through human activities can restrict animal movement, thus reducing overall connectivity (Devictor *et al*. 2008). In areas of continuous, favourable habitat, successful dispersal and subsequent settlement of juvenile koalas (both male and female) is greater than in urban areas with fragmented habitat. This successful dispersal and settlement is largely attributed to a lower rate of juvenile deaths by dog attacks and car collisions (Lassau *et al.* 2008; Tucker *et al.* 2007). If dispersal patterns and social dynamics of koala populations vary across the range based on overall habitat structure, then this is likely to have an effect on genetic structure between regions.

A number of island populations of koala across Australia were sourced from a limited number of founder individuals (Menkhorst 2008). Given the dietary specialisation of the koala, island populations are further at risk of changes to local habitat and stochastic events, as these populations are often isolated. The two northern island populations sampled here displayed diversity levels comparable with

mainland populations (Table 3.1), and returned F_{IS} values close to zero which is consistent with outbred populations (Magnetic Island =0.01, and St Bees Island =-0.03). It appears that the initial founder events (Magnetic Island ≥18 wild individuals, Martin and Handasyde 1999; St Bees Island ~12-17 wild individuals, Lee *et al.* 2012) were not strong enough to reduce genetic diversity below that observed from the mainland. Furthermore, both St Bees Island and Magnetic Island contain large areas of relatively unmodified habitat as a result of limited anthropogenic interference, and this may have contributed to a relatively swift colonisation of islands that would reduce the effect of genetic drift and loss of diversity (Zenger *et al.* 2002). In comparison, the two southern introduced island populations (French Island founded by ~2-3 wild individuals; Kangaroo Island founded by ~18 individuals from French Island, Masters *et al.* 2004) returned positive F_{IS} values (French Island =0.09 and Kangaroo Island =0.19). The Kangaroo Island population is of particular concern, as in addition to it returning the highest inbreeding value, it also has the lowest observed heterozygosity value (Ho=0.09), lowest average standardised individual multilocus heterozygosity (sMLH=0.59), and the highest internal relatedness value (IR=0.83). These populations have been highlighted as having reduced genetic diversity in the past (Cristescu *et al*. 2012; Lee *et al*. 2012b; Taylor *et al*. 1997), with reported cases of physical abnormalities being present in these populations, which is often a result of higher rates of inbreeding (Cristescu *et al*. 2012). This study further confirms the need for careful management of these populations to avoid further loss of diversity. When evaluating mainland populations, inbreeding coefficients were generally close to zero, with the exception of Lismore (F_{IS}=0.11), and the Blue Mountains (F_{IS}=0.1) that returned positive F_{IS} values. However, NetView R clustering indicates that population sub-structuring is present in these population (Figure 3.2), and with relatively high diversity levels, these F_{IS} values are a result of Wahlund effect rather than inbreeding (Christiansen 1988; Sinnock 1975).

The Blue Mountains population appears to hold much of the genetic diversity of the species, with a large proportion of rare alleles being present in the Blue Mountains animals (see also Lee *et al.* 2010; Table 3.1). This is important, as other regions were previously highlighted as key populations for research and conservation to conserve overall species diversity (i.e. South Eastern Queensland; Fowler *et al*. 2000; Lee *et al*. 2012; Ruiz-Rodriguez *et al*. 2014; Wilmer *et al*. 1993), because they were said to have escaped hunting, and thus maintain remnant historic diversity (Cocciolone & Timms 1992; Fowler *et al.* 2000; Lee *et al.* 2010; Lee *et al.* 2012; Ruiz-Rodriguez *et al.* 2014; Wilmer *et al.* 1993). While this may be true to an extent, the Blue Mountains regions (and other areas within New South Wales) appear to have higher diversity levels, and rare genetic variants (Table 3.1). Subsequently, while it is important to preserve all populations of koala, this region should be highlighted for future study if we are seeking to preserve existing diversity for the entire species. Southern populations appear to be less diverse as a whole, and this is likely a result of genetic bottlenecks, translocations, and reintroductions in the past 100-200 years. Comparisons of overall species diversity can be difficult to accurately

estimate, and differences are seen between studies depending on the genetic marker type used, which populations are sampled, and which statistical methods are employed to filter genetic data or estimate diversity (Fowler *et al*. 1998a; Fowler *et al*. 1998b; Houlden *et al*. 1999; Kjeldsen *et al*. 2016; Lee *et al*. 2010a; Lee *et al*. 2012; Lee *et al*. 2010b; Neaves *et al*. 2016; Ruiz-Rodriguez *et al*. 2014). Nonetheless, based on data from this study and Kjeldsen *et al*. (2016), many koala populations display levels of genome wide genetic diversity that are comparable to other outbred animal populations with similar life histories. Furthermore, across koala genetic studies to date, general trends of lower diversity in Victorian and South Australian populations, and higher levels of diversity within New South Wales and Queensland populations, have been widely observed (Cocciolone & Timms 1992; Cristescu *et al*. 2012; Fowler *et al*. 2000; Houlden *et al*. 1996; Kjeldsen *et al*. 2016; Lau *et al*. 2014; Lee *et al*. 2010a; Lee *et al*. 2012a; Lee *et al*. 2010b; Lee *et al*. 2012b; Neaves *et al*. 2016; Taylor *et al*. 1997; Tsangaras *et al*. 2012; Wilmer *et al*. 1993).

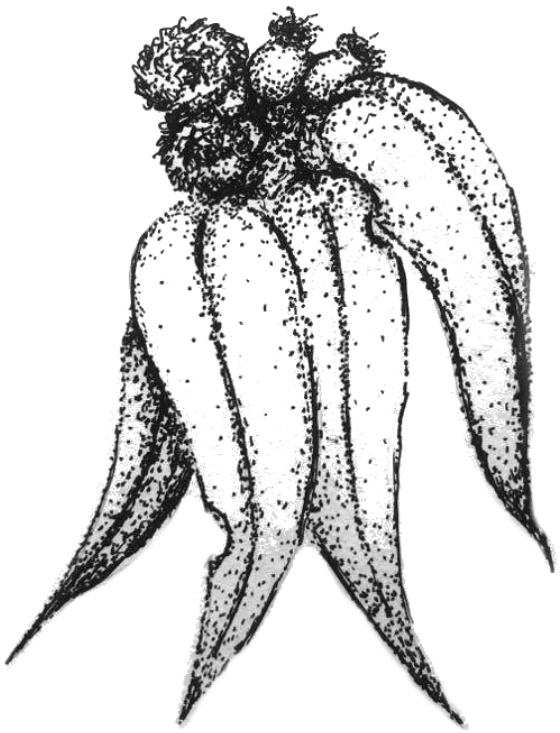
Management Recommendations and Conclusions

Management of species with specialised ecological requirements can be a challenge, given their inherent sensitivity to changes in habitat structure. Classification into appropriate ESUs is crucial for maximizing the evolutionary potential of a species-group, particularly when environmental change threatens the species as a whole. Taxonomic uncertainty can complicate conservation management resulting in potential mixing of different species (or sub-species), which in extreme scenarios can lead to outbreeding depression (Frankham 2003). From a legislative standpoint, legal protection is often defined based on major species groupings, or ESUs (Funk *et al*. 2012), and so resolving these groups accurately is essential to conservation efforts. In the current study, two shallowly divergent phylogenetic clades were observed (Figure 3.6). However, high levels of genetic admixture observed between these clades, particularly at their geographic interface (Sydney Basin region, NSW), and a clear clinal relationship between genetic divergence and geographic location (accounting for 49% of genetic variance; Figure 3.4a), were observed. Furthermore, on a hierarchical level ~70% of the total genetic variance is observed at the individual level, with less than 13% for current sub-species classifications. These results indicate that for the koala, only a single ESU is present, which is in keeping with the most recent mitochondrial research (Neaves *et al*. 2017).

Currently koala populations are managed based on arbitrary geographic distances, with translocations and movement of animals often restricted to local government boundaries, or prohibited completely (Queensland Parks and Wildlife Service 2006; NSW National Parks & Wildlife Service 2001; National Parks South Australia 2016). This management regime is variable across the species range, and perhaps not always ideal to maintain natural genetic structuring. This study indicates that any active management of koalas needs to be considered at a regional level, likely corresponding to environmental bioregions. While no specific regions were identified as showing extreme signatures of local adaptation, much of the selective differentiation observed was accounted for between these bioregions. Likewise,

the strong IBD effect observed in this study indicates that, while a standard arbitrary distance may not be appropriate, geographic distance between populations should be considered, particularly if managing across bioregions.

Across these genetic groups/bioregions, populations containing particularly high levels of genetic variation and diversity should be highlighted in future management plans (e.g. Sydney Basin region – Blue Mountains population). These populations could be considered to be reservoirs holding substantial species diversity. However, the effects of local adaptation between bioregions should not be ignored, because movement of animals into unsuitable habitats may result in overall reduced fitness (Frankham 2003). Despite some regions containing higher levels of diversity, it should be noted that even within a single bioregion, the majority of genetic variance is still accounted for at an individual level, rather than within populations or groups. This variation highlights the importance of conserving koala populations wherever possible. This study gives the most comprehensive genome-wide assessment of koalas, and provides vital information for the informed management of these animals across their range.



Chapter 4: Fine-scale genomic structuring and Environmental Association

Effects of habitat quality/type and social dynamics on population structuring of Koalas (*Phascolarctos cinereus*) across different bioregions

Research Objectives and Chapter Summary

Background/Gap in knowledge

Patterns of dispersal are a fundamental driver of population dynamics and genetic structure within and between populations. Understanding the factors which influence dispersal, and the effects these factors have on genetic structuring, has been an important focus of ecological and conservation studies.

In species' with large and varied ranges, and where social dynamics differ between sexes, assessing environmental effects on genetic structure can allow for more appropriate conservation measures tailored to specific bioregions.

Koalas are considered to be dietary specialists, feeding almost exclusively on eucalypt species. However, their primary food source is found in many different climates, and across several bioregions and habitat structures. Previous studies have indicated that while koalas belong to a single evolutionarily significant unit (ESU), populations show similarities within specific bioregions, and appear to be under similar selective pressures

Aims

i) Assess relationships between biotic and abiotic facts, and genetic structuring of koalas within and across three bioregions.

Significance/Conclusions

In this study, weak levels of IBD were observed between regions, but no strong signatures of IBD were observed within populations. Other environmental factors, or barriers and resistors to dispersal, were more informative than straight-line distances to describe observed patterns of genetic structuring within all regions.

Abstract

Population structure can be affected by a range of environmental and physical factors. These factors primarily affect dispersal capacity, and in turn directly impact levels of gene flow within and between regions. Understanding how these variables impact population structure can assist in the conservation and management of many species. The koala (*Phascolarctos cinereus*) is found across the East coast of Australia, and is found throughout a variety of habitat types (bioregions) and urban areas. To appropriately manage wild populations, we need to understand which factors affect population structuring in this species. Four regions across New South Wales (comprising different bioregions) were sampled to investigate the effects of habitat type, and other environmental variables, on koala dispersal, and population structure. Environmental factors including habitat type (IBRA), variation in rainfall and proximity to a national park (or protected area) were significantly correlated with patterns of population structure (R^2>0.99), and accounted for differences observed between regions. At a distance of <5km, male and female relatedness were similar, although region-specific differences were observed beyond this distance. These differences were largely attributed to degree of habitat fragmentation. Relatedness within 40-50km correlated weakly with geographic distance, but other environmental factors and the effects of barriers to dispersal are more evident beyond this point. Both social structure and environmental factors have a clear impact on population structuring across the range, but patterns are not consistent between regions identified in this study. This indicates that populations may be specifically adapted to each bioregion, and so management plans for this species should consider the level of habitat fragmentation, and overall habitat type of the region of interest.

Introduction

Patterns of individual dispersal are a fundamental driver of population dynamics and genetic structure within and between populations. Understanding the factors which influence dispersal, and the effects these factors have on population structuring, has been an important focus of ecological and conservation studies (Broquet and Petit, 2009). However, the ability to unravel these effects can be difficult, as they often involve complex interactions between a species and its habitat, and can differ across different spatial scales. This can result in different patterns of population structuring across different environments (Broquet and Petit, 2009).

Genetic studies investigating population structuring are often carried out using statistical methods which assume that animals mate randomly, and live within a closed population (Elston *et al.* 2005, Roux, 1974). However, wild populations are rarely so simple. Factors including social structuring, differential sex-biased dispersal, natural environmental features, and anthropogenic features can affect genetic exchangeability across vastly different spatial scales (Gienapp *et al.* 2008). While the effects of these variables can be difficult to quantify, combining available genetic data with information on landscape structure and major physical barriers to dispersal can help to unravel the relationships between physical environment and genetic structure (Manel *et al.* 2003, Storfer *et al.* 2007). This method is broadly known as landscape genetics, and has been a key area of research to help inform fine-scale management of many wild species.

Landscape genetics has traditionally employed small numbers of neutral genetic markers, such as microsatellites or mitochondrial DNA (*mtDNA*) (Manel *et al.* 2003, Storfer *et al.* 2007). However, the increasing availability of genome-wide datasets allows for a much more comprehensive assessment of the genomic relationships between individuals and environmental factors. Genomic datasets provide more accurate estimates of individual relatedness, levels of inbreeding, and overall population structure. They can be used to identify individual sex, and can be used to track sex-biased dispersal. Unlike traditional genetic markers, they are also able to identify signatures of selection across divergent habitats. This is because genome-wide markers provide higher density genomic coverage, sampling regions often overlooked by traditional marker sets (e.g. genomic regions under selection and areas on sex chromosomes) (Liu *et al.* 2005). In species' with large and varied ranges, and where social dynamics differ between sexes, assessing environmental effects on genetic structure can allow for more robust conservation measures tailored to specific bioregions (Henle *et al.* 2004, Shukla *et al.* 1996, Thackway and Cresswell, 1997). Patterns of environmental heterogeneity can change across geographic regions, in turn influencing overall structuring of a population. Variation in structure between regions can arise as a result of several factors, including social structure, degree of gene flow, and local adaptation (Gaggiotti *et al.* 2009). This variation means that populations may need to be managed in different ways. This is particularly important where species exist across large spatial scales, and in different environments.

The Australian landscape has changed dramatically over time, with changes in climate leading to shifts in habitat structures across the continent (Adams-Hosking *et al.* 2011b, Burbidge *et al.* 2009, Petty and Bowman, 2007, Bryant and Krosch, 2016). These changes have led to diverse combinations of habitat types, even within a relatively small geographic region. More recently, significant changes to habitat structures have been seen since European settlement, and large scale land clearing for agricultural or urban development has resulted in a marked reduction in appropriate koala habitat (Kavanagh *et al.* 2007; Melzer *et al.* 2000, Menkhorst, 2008). Given that urbanisation typically introduces a range of physical barriers that can impede animal movement, proximity to urban and suburban areas may affect gene flow on a small spatial scale, and is likely to differ between regions. Other natural barriers to dispersal, such as major waterways and significant geological features, can also present resistance to gene flow within regions.

The koala (*Phascolarctos cinereus*) is an arboreal folivore, which is found along much of the east coast of Australia, across vastly different environments (>80 bioregions) (Burbidge *et al.* 2009). Koalas are considered to be dietary specialists, feeding almost exclusively on eucalypt species (refer to Chapter 1). However, their primary food source is found in many different climates, and across several bioregions and habitat structures. In addition to this, koalas have specific preferences for as few as 3-4 species within a region (Moore *et al.* 2010, Hume, 1999, Martin and Handasyde, 1999, Tyndale-Biscoe, 2005). Previous studies have indicated that while koalas belong to a single evolutionarily significant unit (ESU), populations show similarities within specific bioregions, and appear to be under similar selective pressures (Kjeldsen *et al.* 2019, Kjeldsen *et al.* 2016). While genetic signatures of selection (i.e. local adaptation) have been detected, there has been little study linking these signatures with significant landscape and climate features, or behaviour across different bioregions (Johnson *et al.* 2018, Kjeldsen *et al.* 2019).

At a species-wide scale, geographic distance between individuals and populations has been identified as a key barrier (or resistor) to gene flow (Chapter 3;Kjeldsen *et al.* 2019). Geographic distance is a common factor affecting genetic similarity between populations across large spatial scales, but few studies have looked at smaller scale distances and their potential effect on population structure of koalas between different bioregions. Many studies suggest that anthropogenic effects (including land clearing/modification and urbanisation) have had significant effects on koala population structuring (Abts *et al.* 2018, Beyer *et al.* 2018, Dexter *et al.* 2018, Melzer *et al.* 2000, Menkhorst, 2008). Barriers such as roads/highways, cleared land (e.g. farms), and town developments can interrupt habitat, and make it difficult for animals to move between areas. This can restrict gene flow and carve up natural populations into smaller 'sub-populations' – thus increasing the effects of genetic drift (Burns and Broders, 2014, Medina *et al.* 2012). Natural barriers such as rivers, valleys/rifts and differences in vegetation type/structure can also affect gene flow, however unlike anthropogenic features, these barriers are often stable features of the environment over long periods of time (Capinha *et al.* 2015,

Millions and Swanson, 2007). Similarly, if social hierarchies and dispersal rates differ between ages or sexes, this will affect genetic exchangeability between regions or sub-populations (Dique *et al.* 2003, Gordon *et al.* 1990, White, 1994, Daly, 1981, Singleton and Hay, 1983, Blyton *et al.* 2016).

Koalas are known to have different patterns of sub-adult and adult dispersal, depending on sex (White, 1994). The primary influence for social organisation for koalas is food availability. However, female dispersal and subsequent settlement is driven by access to food resources to increase offspring survival rates, whereas male dispersal is primarily driven by access to mates/receptive females (White, 1994). As a result, male dispersal rates are often higher than in females, although subsequent settlement rates are heavily influenced by environmental and anthropogenic factors. Previous studies indicate that while females tend to stay in areas close to where they were born, the majority of adult males within a given area are immigrants (Gordon *et al.* 1990). This larger dispersal rate in males becomes problematic if they are required to traverse urban areas to find new territory, as there is a higher likelihood of sub-adult mortalities as a direct result of car collisions and dog attacks. Increased sub-adult mortality rates limits potential genetic exchangeability between regions, and can alter genetic structuring within and between populations (Gordon *et al.* 1990, White, 1994).

Identifying genetic structure alone can be inherently informative, however incorporating environmental data can help to explain the underlying drivers of this structure (Manel *et al.* 2003, Storfer *et al.* 2007). Significantly, these relationships provide further information concerning behavioural and social ecology of a species, and how environmental and anthropogenic barriers can influence genetic structure (Millions and Swanson, 2007). Understanding these drivers is important as it provides tangible cornerstones for conservation management. In this study, using a combination of genome-wide SNP markers, coupled with publicly available environmental data, I investigate the relationships between population genetic structure of koalas and environmental factors, and how these may differ between sexes, across three distinct bioregions, and four populations. I also investigate if these relationships differ between bioregions, and spatial scales.

Methods

Sampling strategy and Genotyping

To compare patterns of population structuring between bioregions, and between habitat sub-types within bioregions, a total of 198 individuals from four geographically distinct populations, corresponding to three bioregions, were used for this study (Figure 4.1; Table 4.1). Each sample was required to have accurate GPS coordinates from the sampling location, so that environmental data could be assigned to each sampling point. Samples were acquired opportunistically and were genotyped (SNP markers) using a DArtSeq™ approach as described in Kjeldsen *et al.* (2019). The sample utilised in this study were sourced from a number of existing studies, and were obtained originally as part of routine veterinary care, or active monitoring programs (Crowther *et al.* 2014; Griffith *et al.* 2013).

To ensure SNP markers were informative and of high quality in the four populations sampled, SNPs were filtered based on >70% call rate and a MAF >0.05 across all samples. Sex-linked markers were also identified based on the criteria outlined in Kjeldsen *et al.* 2016. In order to ensure confidence in putatively identified X- and Y-linked SNPs, these markers were identified within a subset of individuals with known sex (n=102), and then tested against an independent subset (n=25) of individuals with known sex. If a SNP correctly identified the sex of an individual, it was deemed appropriate for diagnostic/sex assignment purposes.

Once sex linked SNPs were identified and tested, these were then used to assign sex to individuals without existing sex information. In order to assess potential differences in sex-biased dispersal, genotypic datasets were then divided into three subsets; all individuals, males only, and females only.

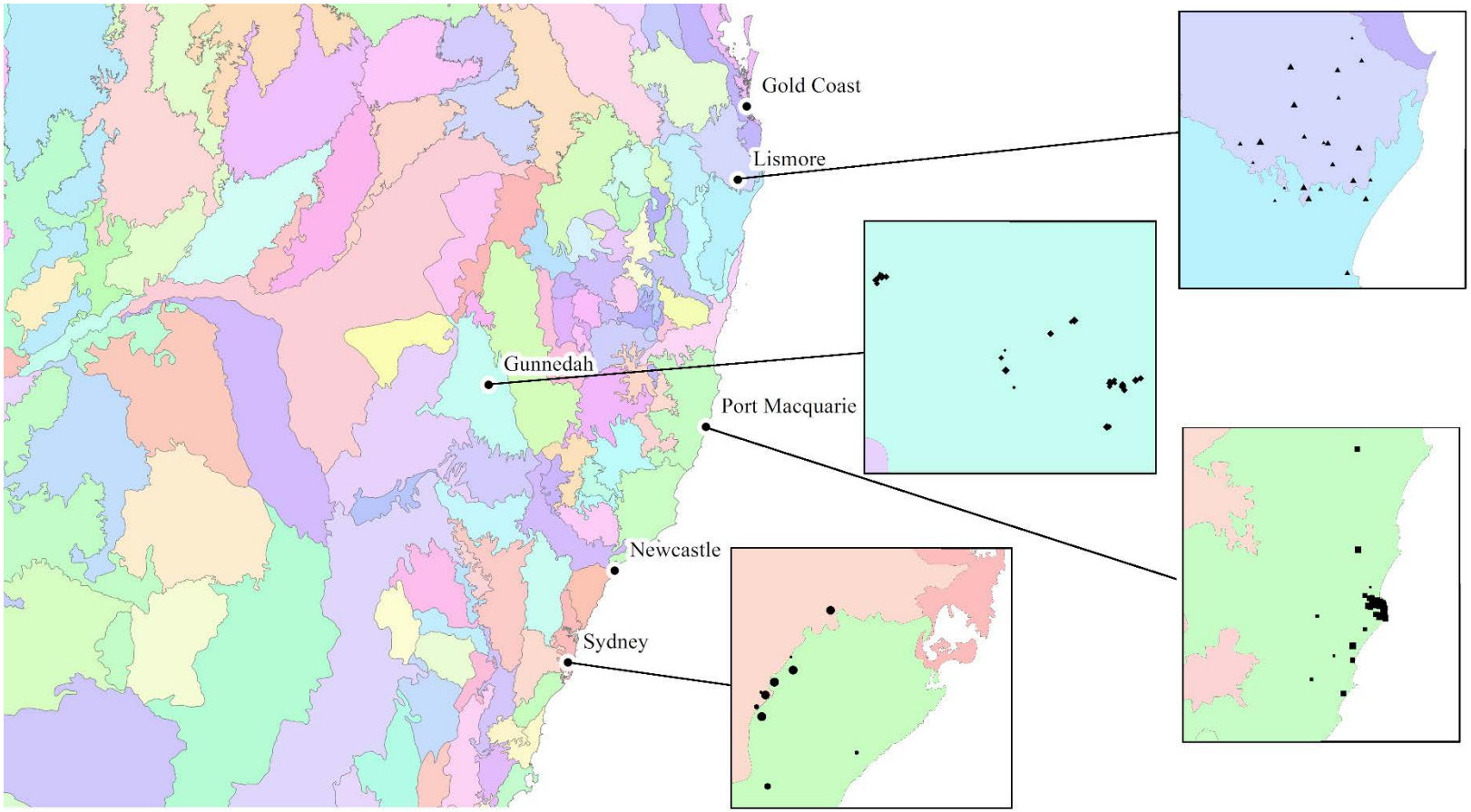


Figure 4.1. Sampling map showing distinct bioregions – colours are arbitrary, but are representative of different habitat subregions as per the Interim Biogeographic Regionalisation for Australia map (IBRA). Black markers indicate relative sampling density, with larger markers indicating more than one sample was obtained from this geographic point.

Table 4.1. Samples and average values for all environmental variables. Mean annual values indicate average low and high values across 30 years. ⸸

Population	n	Bioregion (IBRA)	Habitat subgroups surveyed	Largest distance between 2 samples (km)	Annual Temp (°C)	Annual Rainfall (mm)	Annual Rainfall Variation	Mean Elevation (m)	Mean Slope	Mean distance from water bodies (km)	Mean distance from national Parks (km)
Lismore	33	NNC	7	600	8-20	607-2912	66.48	89.68±98.07	3.35 ± 2.67	0.02±0.018	0.06
Port Macquarie	73	NNC	20	150	8-20	607-2912	73.83	35.88±120.87	3.07 ± 2.34	0.02±0.014	0.48
Gunnedah	36	BBS	22	50	10-19	449-1015	174.30	308.28±15.41	1.47 ± 1.60	0.01±0.008	0.11
Sydney Basin Region	56	SYB	7	250	10-17	522-2395	177.29	154.94±118.83	2.35±1.14	0.01±0.006	0.04

⸸ Bioregion (IBRA) refers to the overarching bioregion/habitat type as originally described in (Thackway and Cresswell, 1997). Habitat sub-groups refer to habitat types within each of these larger regions, refer to https://www.environment.gov.au/land/nrs/science/ibra for further information.

Sampling Regions, Landscape, Environmental and Anthropogenic factors

For this study, a number of geospatial, environmental and anthropogenic factors were assessed for their relationship to genetic structuring and relatedness patterns. Each sampling point was assigned a value or category based on exact sampling location (latitude, longitude), bioregion (IBRA), habitat sub-type (biogeographic sub-region), mean annual temperature range, mean annual rainfall, mean elevation, mean slope, mean distance from major freshwater water body (including rivers and lakes), and proximity to a urban or suburban settlement (Table 4.1). This data was collected and extrapolated from several publicly available databases (see Supplementary Material 4.1), with the use of ArcGISPro mapping software (Scott and Janikas, 2010).

Fine-scale genetic sub-structuring (individual relatedness)

To assess fine-scale relatedness within regions, and to identify any possible relationships between individuals, several relatedness measures were examined (listed below). Individual relationships were estimated by a 1–proportion of shared alleles (1-PSA) matrix, using the *propShared* function in the package *adegenet* in R. In addition to this, other relatedness measures were calculated between individuals using the program Co-ancestry (Wang, 2011), including Lynch & Li's genetic distance, Ritland's genetic distance, and Wang's Genetic distance (Supplementary Table 4.2).

To investigate if dispersal patterns were different between regions, and between sexes, spatial autocorrelation analysis was performed in Genalex v6.502 (Peakall and Smouse, 2006), using Ritland's genetic distance metric. Each region was tested individually, using distance classes of 500m up to the maximum distance within regions, and 1,000 iterations. Additionally, as overall distance varied between regions, tests using distance classes of 1km, up to a maximum of 100km were also performed. Sex biased dispersal was assessed in all populations by splitting each population into putative male and female groups. Where sex was not recorded with the original sample, each sample was assigned as male or female based on putatively sex associated markers.

To test if individual relationships was correlated with geographic location, an F-test was conducted comparing geographic location, and individual relatedness values in Genalex v6.502 (Peakall and Smouse, 2006). Isolation by distance (IBD) mantel tests were conducted both within and between populations, by comparing 1-PSA values on all loci, with geographic distance matrices. Geographic distances used for these tests were calculated based on straight-line distances. All sample locations were approximate based on where each individual animal was found, and despite numbers of males and females being represented within this dataset, samples obtained as a result of injury by car accident or dog attacks may have a skewed sex ratio due to higher likelihood of juvenile/sub-adult males being injured as a result of roaming.

Population-level genetic structuring (average relatedness)

To further assess genetic structure between individuals within and between regions, multiple genetic distance measures were calculated. Genetic relationships between sub-groups within and between regions were assessed using several approaches, including Weir and Cockerham's unbiased F-statistic (Fst) (Weir & Cockerham 1984), Nei's unbiased genetic distances (Nei 1978) in Genalex (Peakall and Smouse, 2006) (Supplementary Table 4.2). Genetic distance calculations were based on all polymorphic SNPs.

Genetic clusters within each region, and between all regions sampled were calculated through an IBS distance matrix in PLINK (Purcell *et al.* 2007), and subsequently visualised using the NetView R program (Neuditschko *et al.* 2014; Steinig *et al.* 2015) at multiple k-NN values (k-NN = 1-50). Optimisation of k-NN values was performed by plotting each k-NN value against the number of communities detected using a "Fast-greedy" clustering algorithm. Similarly, a principle component analysis of genetic factors was conducted on the dataset using DAPC in *adegenet* (Jombart, 2008).

Gene flow and Environmental Factors

Both continuous and categorical variables were assessed in this study (Table 4.1, Supplementary Table 4.1). Categorical variables were mostly associated with bioregions or habitat sub-types (Table 4.1), and were analysed using either a "presence/absence" approach, or by ranking each sub-variable to describe its suitability to koala biological requirements (i.e. shelter, food and water). This approach was based on a combination of several habitat quality ranking approaches, but was informed predominantly by the presence of potential food trees within a given area (Rossi and Kuitunen, 1996, Callaghan *et al.* 2011). To simplify this process, a decision tree was constructed (Figure 4.2), where desirable traits were allocated a positive value (+1), and non-desirable traits were allocated a zero score. The cumulative score for each sub-variable was ranked from "best" to "worst". For categorical predictors, including bioregion (IBRA), habitat sub-type (HT), and habitat class (HC), each category present within the data was ranked according to "suitability for koalas within a given region". Briefly, each category was evaluated for the presence or absence of potential food trees (based on recorded distributions of these tree species), then if potential food trees were present, the presence or absence of preferred food trees was noted (Office of Environment and Heritage, 2018). This approach was used in an attempt to normalise habitat suitability across geographic regions, and account for the fact that even though tree species may differ between regions, these differences may not impact the koala's access to resources. Further to this, fragmentation in each region was estimated, with highly fragmented areas being assigned a lower suitability score. In each case, these factors were categorised as "high/common" or "low/uncommon", to take into account variation within each factor. The final result produced a ranked scale from "most suitable" to "least suitable" habitat types for each category. These values were then used in downstream association analyses (Figure 4.2).

Associations between environmental variables were identified using the *pair.panels* function in *psych* (Revelle and Revelle, 2007), and any correlations $R^2 \geq 0.7$ between environmental factors were deemed highly linked, and only a single factor/variable was retained to reduce biasing results. Genetic associations between single or multiple environmental factors were assessed using two statistical approaches; a latent factor mixed model (*lfmm*) approach in the R package *Vegan* and LEA (Frichot and François, 2015), and a redundancy analysis (RDA) approach in the R package *RDA* (Oksanen *et al.* 2013). For *lfmm* analysis, each environmental variable was run as a fixed effect, while genetic structuring data represented latent factors in the model. Multiple models were run, assuming between one and six populations were present (based on clustering observed in DAPC and NetView plots). An FDR correction (FDR = 0.1, 0.05, 0.01 and 0.005) was applied to all resultant p-values to determine an appropriate threshold for significance.

The redundancy approach (RDA) used here is a multivariate ordination technique which allows for all parameters/variables (genetic, individual and environmental) to be considered simultaneously. Similar to the approach used in DAPC analysis in *adegenet*, this approach identifies groups of markers which are associated with specific environmental variables. Here, a simple additive model was used, where the sum of all environmental variables equated to the genetic variance observed:

$$gt \sim HT + IBRA + HC + Elev + Slope + Water + Rainfall + Rain\_var + Nat\_Park$$

Gt = Genotype
HT = Habitat Type
IBRA = Bioregion (Thackway and Cresswell, 1997)
HC = Habitat class
Elev = Elevation (m)
S = Slope
Water = Proximity to water body (fresh water)
Rainfall = Average Annual Rainfall

In order to identify markers which are associated with each variable, RDA loadings were plotted, and any markers which were placed at the tail ends of the distribution (≥3σ from the mean) were deemed "outliers".

Once candidate markers were identified in both methods, the sequences associated with these SNPs were compared with a public database (https://www.ncbi.nlm.nih.gov/), and were matched to the koala genome (RefSeq GCF_002099425.1) to assess if they were possibly associated with functional regions of the genome.

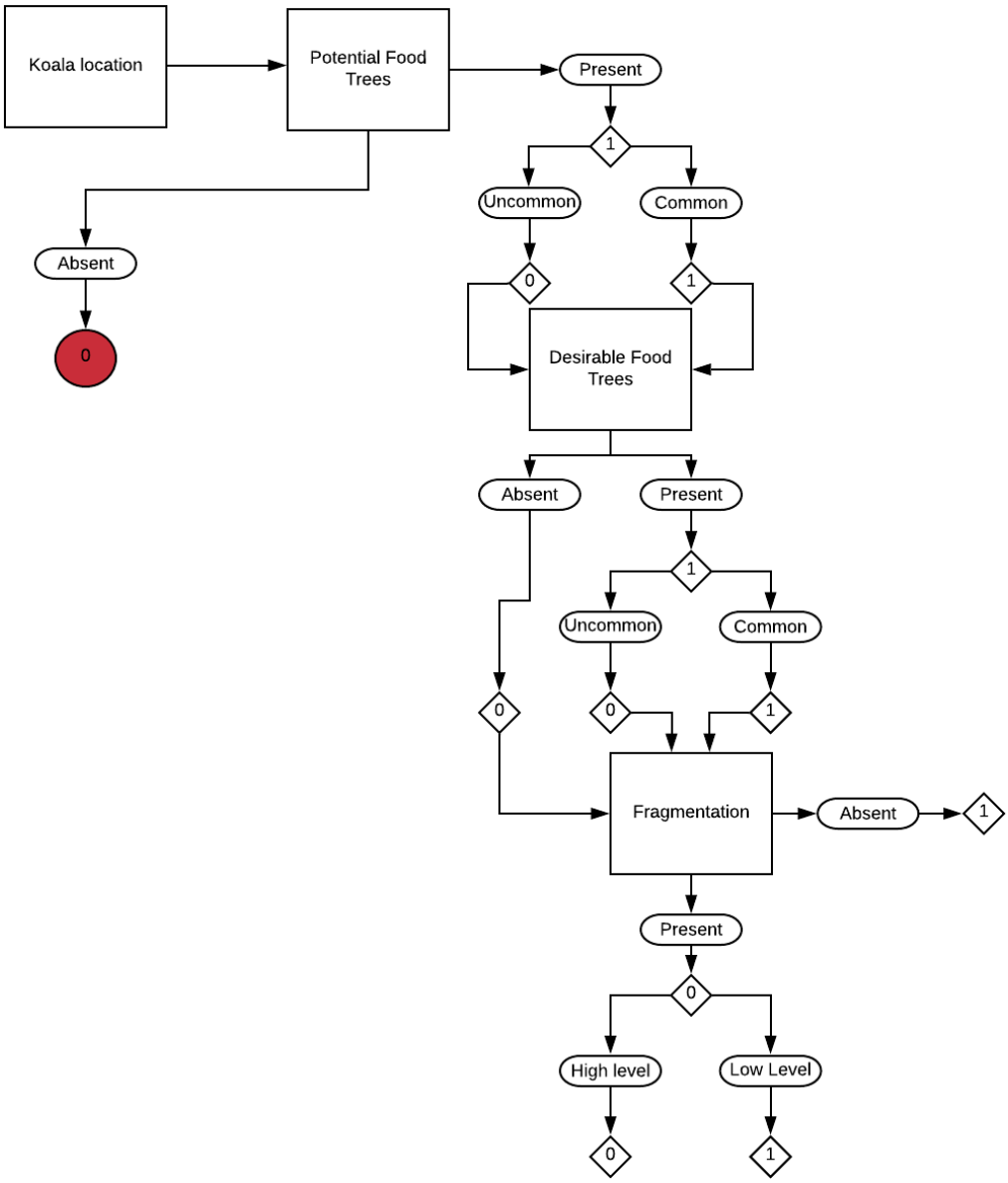


Figure 4.2. Decision tree for ranking habitat within each region. Approach based on and adapted from the National Koala Conservation Management Strategy (National Resource Management Ministerial Council, 2009), and as described in Callaghan *et al.* (2011) and Rossi and Kuitunen (1996).

Results

Fine-scale genetic sub-structuring (individual relatedness)

Genetic sub-structuring was identified in all study populations through plotting genetic distances (1-PSA) in NetView R at kNN=10-20 (Figure 4.3). Animals from the Port Macquarie region displayed clear sub-structuring, clustering into two major groups, largely corresponding to their location in relation to a major river system which splits the region (Port Macquarie River). Similarly, both the Lismore and Sydney populations displayed sub-structuring into two distinct genetic clusters. No clear sub-structuring was observed in the Gunnedah population, with all individuals clustering closely in both NetView R plots, and returning high average relatedness (Supplementary Table 4.2). These patterns of sub-structuring generally could not be explained by geographic distances alone.

Average relatedness (Ritland 1996) within populations ranged from 0.00-0.71 (SD± 0.14), being highest in Gunnedah. Internal/individual relatedness (IR) and inbreeding (F_{IS}) ranged from 0.49-0.63 and 0.03–0.11 respectively, and varied both across populations, and within regions (Chapter 3 Table 3.1). Sydney showed the lowest average levels of inbreeding (av F_{IS} = 0.03±0.00).

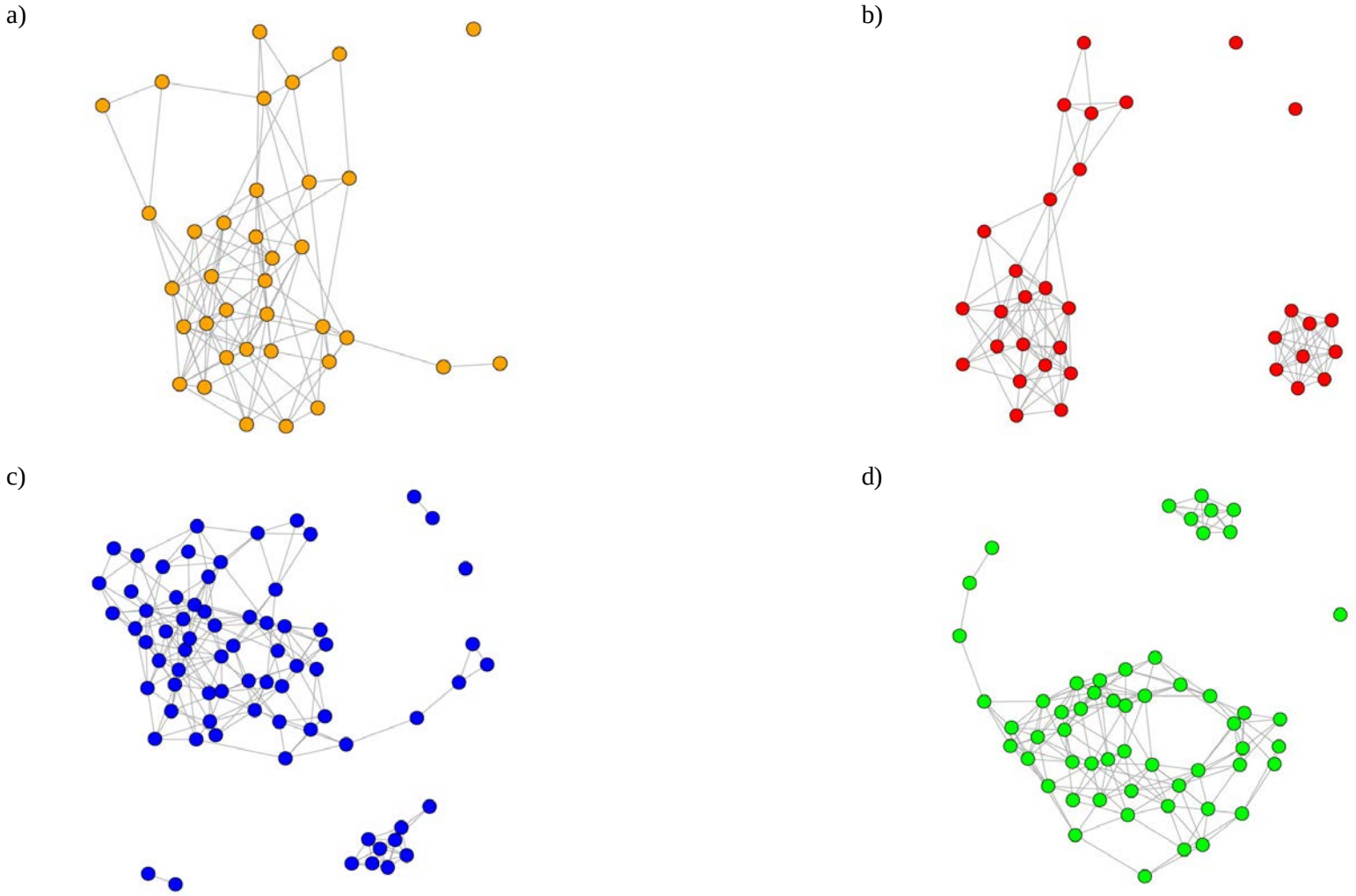


Figure 4.4. NetView plots displayed at k-NN =10 a) Gunnedah, b) Sydney, c) Port Macquarie and d) Lismore.

Population-level genetic structuring (average relatedness), Gene flow and Geographic Distance

At a large geographic scale (between regions or populations), average gene flow appears to follow an isolation-by-distance dispersal model (R^2=0.69), however only weak signatures of IBD could be observed within regions when direct physical distances (km) were used (Figure 4.5). When IBD within each region was examined, the only regions which displayed significant IBD effects at a regional level were the Sydney Basin, and Lismore region, however even these were relatively low (R^2=0.228 and R^2=0.508 respectively). Surprisingly, when the sexes were separated within each region, males and females showed opposite trends in the Gunnedah sampling region, with males showing higher relatedness over larger geographic distances compared to females (Figure 4.5). It should be noted however that, as a result of opportunistic sampling from animals taken into veterinary care, samples may have been skewed towards a particular demographic (eg. sub-adult males).

Interestingly, while no major IBD effects were observed in the study regions, there were also no observable patterns when taking overall sampling distance into account before outlier individuals were removed – i.e. Gunnedah, which had the smallest sampling area (~50km^2) showed the similar IBD correlations to Lismore (~600km^2), indicating that distance alone could not account for all genetic variation seen. Overall, Lismore and Sydney displayed similar patterns up to 80km, where a slight IBD effect was seen. Port Macquarie and Gunnedah did not show any obvious IBD effects, however levels of genetic differentiation differed significantly between these regions (Figure 4.5).

Similarly, spatial autocorrelation did not reveal any significant differences across all populations, however when each population was examined up to 50km (maximum distance between samples at Gunnedah), Lismore showed a distinct decline in relatedness between individuals further than ~17-20km apart, which was not observed in the other populations (Figure 4.6). This pattern was also observed in DAPC and NetView results (Figure 4.3), where two distinct genetic clusters were observed in this population.

Clear sex biased dispersal patterns were observed in all populations sampled, based on both mantel tests, and spatial autocorrelation results. In all cases, it appears that in distances <5km, there was no obvious differences between male and female relatedness, however average relatedness reduced beyond this point (Figure 4.6).

It should be noted that the samples obtained from the Sydney Basin region (which also passed quality thresholds) were all assigned as female. While this is statistically unlikely in a random sampling approach, as these samples were opportunistically obtained, it is possible that the sampling method in this region may have been biased towards female koalas.

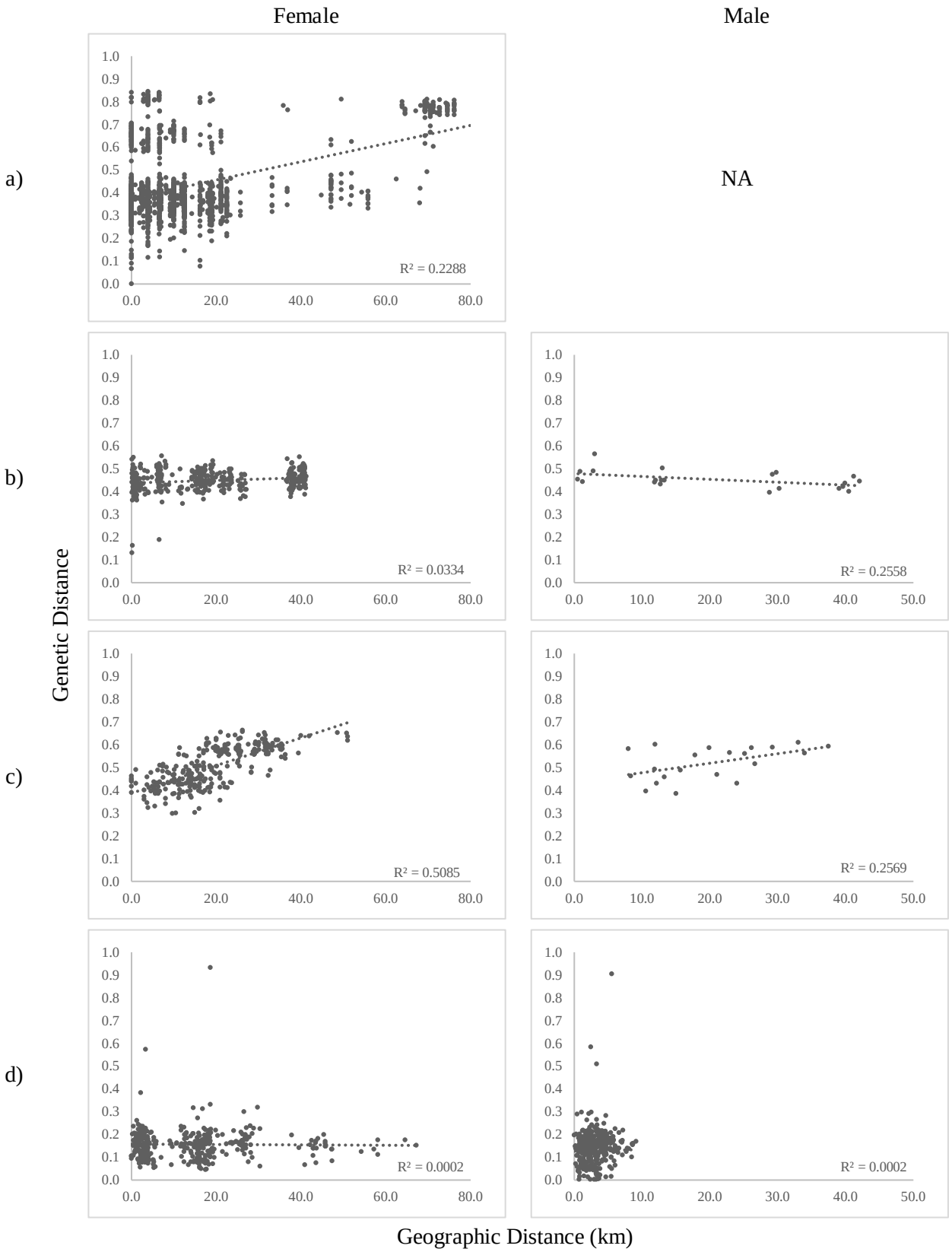


Figure 4.5. Differences in IBD between sexes using Ritland's genetic distance metric (variable distances up to 80km). a) Sydney, b) Gunnedah, c) Lismore, and d) Port Macquarie.

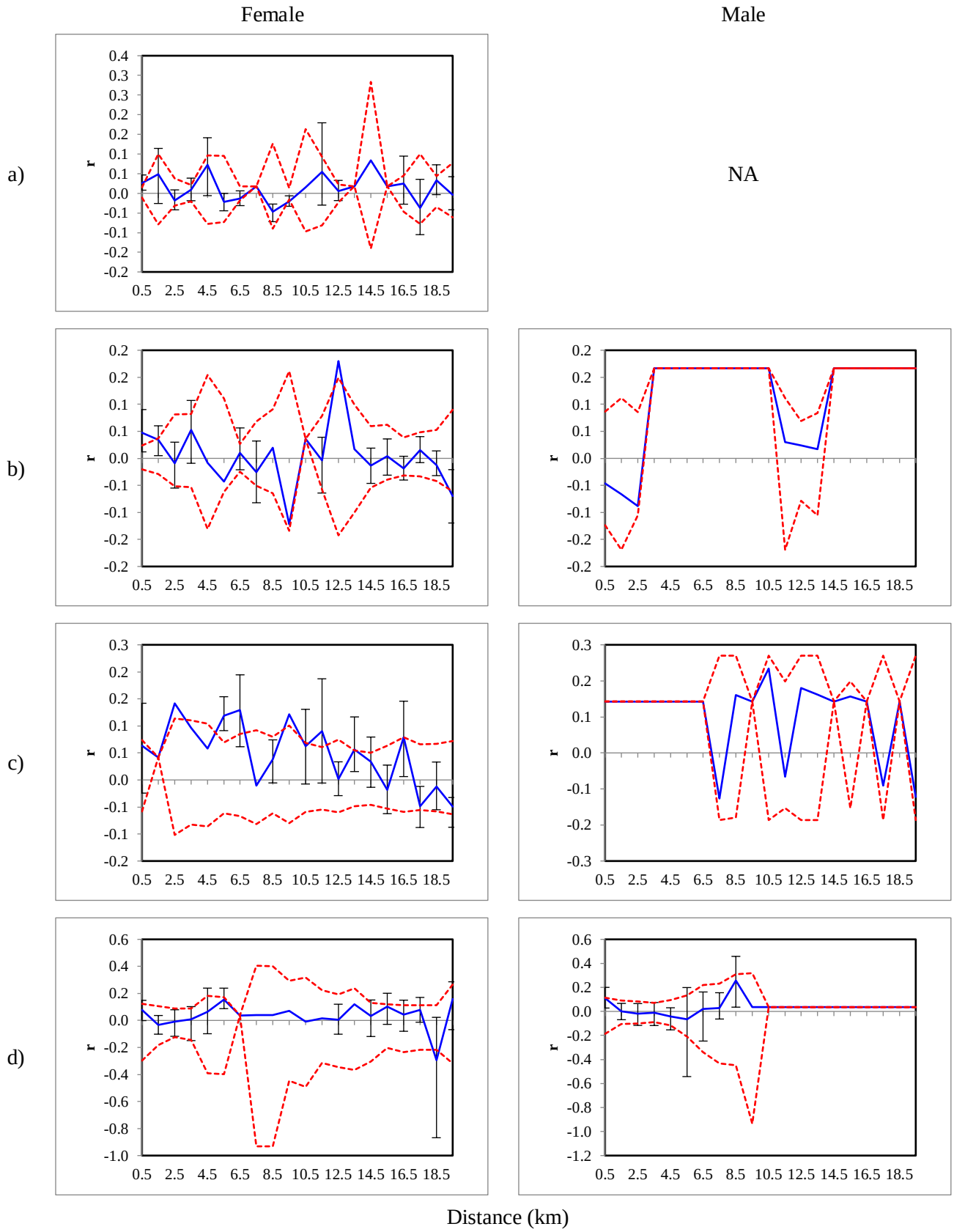
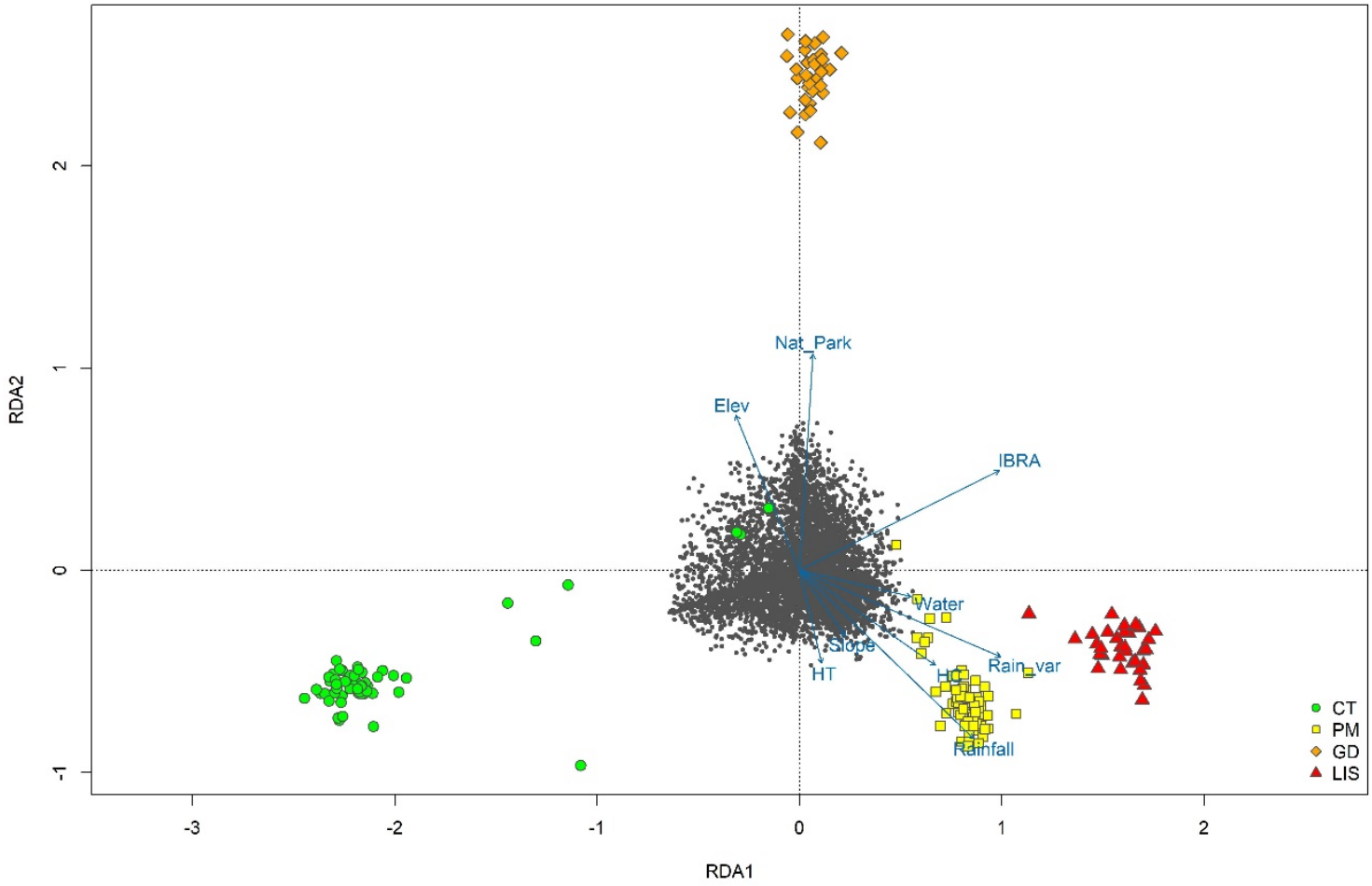


Figure 4.6. Sex biased dispersal (spatial autocorrelation). Standardised to 20km, and a class distance of 1km. a) Sydney, b) Gunnedah, c) Lismore and d) Port Macquarie.

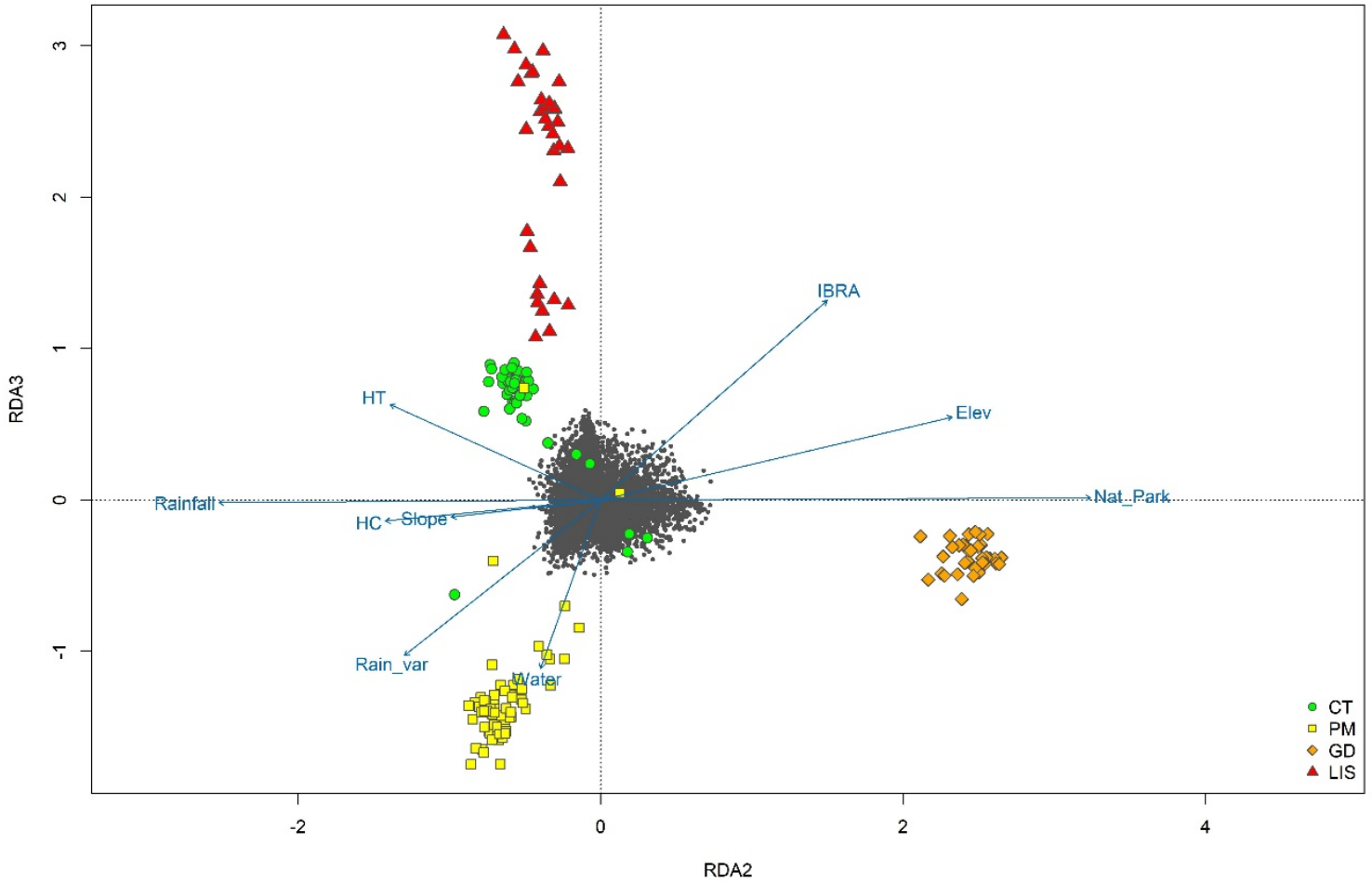
Gene flow and Environmental Factors

The observations made in the IBD analyses (Figure 4.5) indicate that factors other than purely geographic distance may be affecting patterns of genetic structure within and between regions. Several environmental variables were investigated to try to explain these patterns. Correlations between environmental variables ranged from -0.66 to 1.00 (Supplementary Figure 4.1), with Habitat Form (HF) and Habitat Class (HC) being highly correlated (R^2=0.9), and Average Annual Rainfall (Rainfall) and Average rainfall during the Wet/Dry season (Wet/Dry) being highly correlated (R^2=0.99-1.00). As a result of these variables being highly linked, HF, Wet, and Dry variables were removed from further analysis. Average variation in rainfall (Rain_var) was also correlated with Average Annual Rainfall (R^2=0.81-0.85), however this metric was retained in downstream analysis to determine if there was a difference between consistence of rainfall in each area.

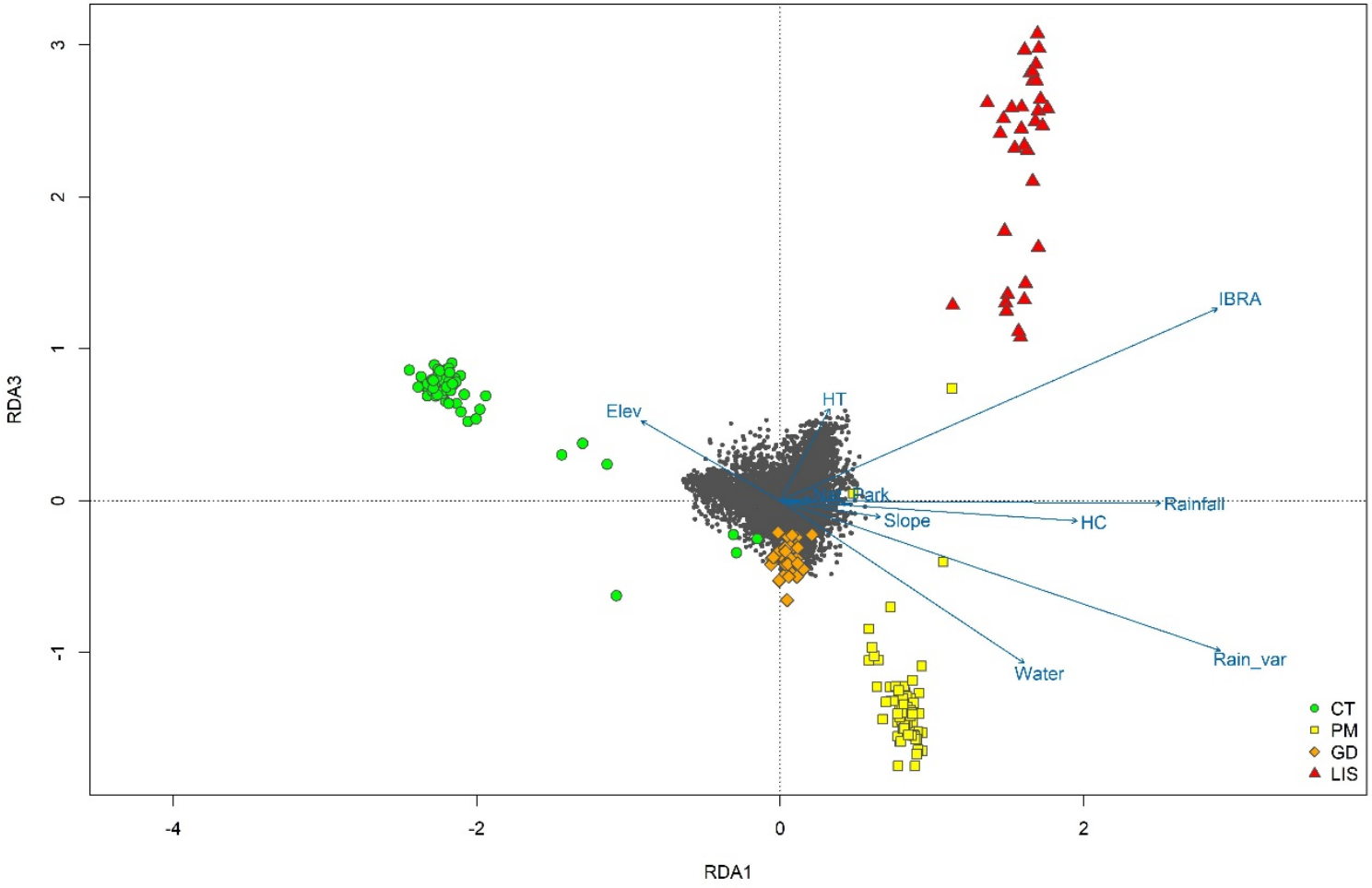
Markers correlated with patterns of genetic structuring were identified for all environmental variables, with Rainfall variation, Bioregion type (IBRA), and proximity to a national park having the most significant relationships when observing the first two principle components in RDA, and in *lfmm* analysis (Figure 4.7). Proximity to a national park was associated with the most genetic variance (an average of 55% of genetic variance), and the broader habitat class (HC) explained the least amount of variation in genetic data (21%). A total of 308 unique markers were identified as being significantly associated with environmental predictors (Table 4.2). Interestingly, in all but the first two principle components, Lismore clustered again into two genetic subgroups, which appeared to be associated with bioregion (IBRA). The highest numbers of predictive markers were associated with proximity to a national park (RDA = 82 SNPs), Bioregion sub-type (RDA = 59 SNPs, *lfmm* = 80 SNPs), and with rainfall variation (RDA = 42 SNPs, *lfmm* = 51 SNPs). All markers which were significantly correlated to an environmental variable were aligned to the koala genome and transcriptome, however no markers were able to be identified as being functional.


RDA2
RDA1
-3
-2
-1
0
1
2
Nat_Park
Elev
IBRA
Water
Slope
HT
Rain_var
Rainfall
CT
PM
GD
LIS

a)


RDA3
RDA2
3
2
1
0
-1
-2
0
2
4
IBRA
HT
Elev
Rainfall
Nat_Park
HC
Slope
Rain_var
Water
CT
PM
GD
LIS

b)


RDA3
RDA1
3
2
1
0
-1
-4
-2
0
2
IBRA
HT
Elev
Rainfall
Slope
HC
Water
Rain_var
CT
PM
GD
LIS

c)

Figure 4.7. a, b, and c. RDA plots depicting relationships between environmental variables and individuals (coloured markers) or individual SNPs (grey dots). Coloured markers indicate individual samples, and their genetic similarity to one another – markers which are closer together represent individuals with more genetic similarities. The location of these marker in relation to each environmental predictor indicates a positive or negative relationship. e.g. RDA3 shows a strong positive relationship with samples from Port Macquarie, indicating that these individuals are found closer to bodies of water. SNP markers are represented by grey dots, and where these tend towards an environmental predictor (indicated by an arrow), these groups are assumed to be positively correlated with that specific predictor. Longer arrows are indicative of stronger correlations. CT – Sydney, PM – Port Macquarie, GD – Gunnedah and LIS – Lismore.

Table 4.2. Number of SNPs identified to be putatively associated with each environmental variable. RDA approach deemed markers to be outliers at 3σ from the mean, while an FDR correction (FDR = 0.1, 0.05, 0.01 and 0.005) was conducted in the *lfmm* approach. FDR=0.005 calculated using a conservative algorithm (Benjamini-Hochberg) is presented here. Average, Min and Max Corr indicate the correlations between each environmental predictor, and explained genetic variance.

Predictor	Method	Number of SNPs	Average Corr	Min Corr	Max Corr
HT	*lfmm*	-			
	RDA	17	0.23	0.17	0.32
IBRA	*lfmm*	80			
	RDA	59	0.37	0.12	0.67
HC	*lfmm*	32			
	RDA	11	0.22	0.12	0.40
Elev	*lfmm*	6			
	RDA	41	0.24	0.12	0.36
Slope	*lfmm*	16			
	RDA	20	0.23	0.14	0.31
Water	*lfmm*	19			
	RDA	24	0.25	0.14	0.44
Rainfall	*lfmm*	26			
	RDA	12	0.30	0.10	0.59
Rain_var	*lfmm*	51			
	RDA	42	0.32	0.13	0.67
Nat Park	*lfmm*	23			
	RDA	82	0.55	0.20	0.78

Discussion

Previous studies have indicated that at a species level, genetic structuring of koala populations is likely driven by an isolation by distance (IBD) dispersal model (R^2=0.695) (Kjeldsen *et al.* 2018). While this is important to consider for management at a broader level, this pattern does not appear to hold true at a smaller, regional scale (R^2= 0.001–0.158). In this study, only weak signatures of IBD were observed within and between regions, and these were highly variable, indicating that factors other than geographic distance are influencing genetic structure. Other environmental variables (and/or barriers to dispersal) were more informative than simple geographic distances to explain overall genetic structure within all regions. Similarly, relatedness patterns differed between sexes, indicating behavioural (dispersal) differences between males and females. Population structuring is likely to be a result of complex interactions between several biotic and abiotic factors, however the most significant influencing factors appear to be directly linked to habitat type.

Gene flow and population structure between regions

Koalas are known to be relatively sedentary animals, sleeping for up to 18 hours each day (Martin and Handasyde, 1999, Tyndale-Biscoe, 2005). Previous studies have indicated that koala home ranges vary considerably, and can range from 4-40 hectares (0.04–0.4km^2), with male dispersal being higher on average than females (White, 1994). Female koalas are also more likely to remain close to where they were born, with sub-adult males being more likely to roam further to establish new home ranges away from their mothers (Dique *et al.* 2003, Norman *et al.* 2019). Dispersal is primarily driven by the need for resources (e.g. food, shelter, or mates), and so can be influenced by factors such as population density, and habitat availability and continuity (or lack thereof) (Gordon *et al.* 1990, White, 1994). If no barriers to dispersal are present, mating is random, and all biological needs of the koalas are met, we would expect to see a high correlation between geographic distance and genetic structure (i.e. a strong IBD effect). While this can be seen at a species level (as seen in Chapter 3), at a regional spatial scale (i,e. distances of 400–1000 km), genetic differentiation of koala populations does not significantly correlate to simple geographic distance (Figure 4.5). Additionally, there are differences in the effect of geographic distance on genetic differentiation between regions, with some regions showing moderate levels of IBD, and others showing negligible levels over the same spatial scales (Figure 4.5).

Within a range of 5km, all populations show similar patterns of genetic relatedness (Figure 4.6), with average pairwise relatedness reducing over increased distance. This distance is consistent with the minimum sizes of home ranges reported for female koalas, with individuals within this range being expected to have high relatedness (Goldingay and Dobner, 2014). At a straight line distance of 15–20km, pairwise relatedness in all populations reduced markedly, with another distinct reduction in pairwise relatedness observed at 28km (Figure 4.6). Males from the Gunnedah population were an exception to this, in that relatedness between individuals appeared to increase over larger geographic distances, however it should be noted that this was only a slight increase. Habitat appears to be more

fragmented within this region, and so it is possible that this pattern of relatedness reflects a tendency for males to disperse more readily in this region in search of resources (Lunney *et al.* 2012). Gunnedah is a largely cleared or modified landscape, with habitat corridors present along ridgelines throughout the region. These corridors may facilitate gene flow within this region, resulting in the low IBD effect reported here. Interestingly, while both the Gunnedah and Port Macquarie sampling sites displayed the lowest IBD effects, genetic distance estimates were vastly different between these regions (Average genetic distance = 0.44 and 0.16 respectively).

Higher dispersal and subsequent settlement of males would lead to higher gene flow, and smaller IBD effect. In the Port Macquarie region, a slight IBD effect was observed beyond approximately 50km (R^2=0.088), with average relatedness otherwise being consistent across spatial scales (Figure 4.5). Differences were observed between males and females however, with relatedness diverging beyond a distance of 4km (Figure 4.6). Interestingly, the Lismore population showed the lowest IBD effect when all samples were considered (R^2=0.0001), despite having the largest overall sampling range. Lismore also did not follow the same relatedness patterns as the other populations, with average relatedness increasing initially up to 17-20km, and then dropping distinctly after this. This was again reflected in genetic clustering analysis in NetView, which revealed sub-structuring into two groups. When these sub-groups were analysed separately (up to 80km range), a higher IBD effect was observed in both males and females (R^2=0.25 and 0.50 respectively). The stronger IBD effect observed here also indicates that population structuring at a small spatial scale may be influenced by geographic distance, but that over larger distances (>40km), barriers to dispersal, or other factors may significantly affect genetic exchangeability.

In three of the populations studied here (Lismore, Port Macquarie, and Sydney), clear genetic sub-structuring was present within regions (Figure 4.3). This sub-structuring, coupled with weak IBD effects, indicate that other environmental factors are responsible for much of the patterns seen in koala populations sampled in this study. One exception to this is koalas from the Sydney region, which displayed higher IBD effects, and the lowest overall individual relatedness (but highest variability) at a smaller scale (<50km). Generally the animals from within the Sydney region displayed less variability between individuals than from other regions, with the exception of a small group of individuals sampled from the southern areas surrounding Sydney, and from closer to the east coast. These individuals are physically separated by large areas of relatively undisturbed habitat - the Sydney Royal National Park (Benson, 2008). While the habitat within this area is largely considered favourable to koalas, being mostly Eucalypt woodlands with areas of tussock grass understorey, this may also mean that koalas may not need to disperse as far as in urban or cleared areas in order to find reliable resources, leading to small pockets of higher relatedness (Kreuzer and Huntly, 2003). This pattern has been seen in other arboreal species, where dispersal relies heavily on habitat corridors to facilitate genetic exchange between regions (Bright, 1998, Laurance, 1990).

Environmental Factors

Environmental factors are often interlinked and reliant on each other, which can make untangling the relationships with population structure difficult (Guillot *et al.* 2014, Jiménez-Valverde *et al.* 2009). Here, several environmental factors were closely correlated to one another (Supplementary Figure 4.1), particularly when climate variables were compared to habitats and bioregions. Physical factors such as elevation can often be linked to other environmental variables such as temperature and rainfall, which can in turn affect habitat structure. Likewise, habitat structure and composition will invariably be linked to other physical factors such as soil type and rainfall. Higher rainfall is often associated with denser vegetation coverage, with variation in elevation additionally providing microclimates within a region, which foster higher diversity (Varner and Dearing, 2014). In the populations sampled, rainfall was grouped into two overarching categories, with Gunnedah and Campbelltown having lower average rainfall throughout the year, and also less variation in rainfall, and Lismore and Port Macquarie having both higher overall rainfall, and more variability throughout the year (Table 4.1). Correlations between genetic structure, and both total annual rainfall, and rainfall variability were reflected this pattern when assessed using environmental association analysis (Figure 4.7).

The koala is a dietary specialist, and as such is restricted to habitats/bioregions able to support appropriate eucalyptus species. The region-specific dietary preferences may be due to a number of factors, and has been suggested to be linked to both the overall chemical profile of leaves, and to specific microbiome characteristics in each region (Dique *et al.* 2003, Varner and Dearing, 2014). Previous studies have suggested that koala populations are under some degree of selective pressure associated with changes in habitat and bioregion (Kjeldsen *et al.* 2019). The Lismore sampling region not only spanned the largest geographic area, but also encompassed two distinct bioregions; SEQ and NNC. In addition to several bioregions being sampled, Lismore was more variable in habitat sub-type, with samples being found in both residential areas, and agricultural areas. Genetic sub-structuring analysis showed two genetic clusters (Figure 4.3) corresponding to these habitat types. Lismore koalas also displayed the strongest positive correlation to bioregion (IBRA), further supporting habitat as a key driver of genetic structure. Samples collected from the Sydney region are also located along the border of two different habitat types, and as such, are moderately associated with bioregion (Figure 4.7). However, some of these samples do not follow the same trend, indicating that habitat alone could not explain genetic structuring in all regions. This is important to note, as it indicates that environmental factors do not influence genetic structure universally between regions. Studies have also suggested that habitat fragmentation may be a driving force behind observed population structure for these animals (Dexter *et al.* 2018, Dique *et al.* 2003, Kjeldsen *et al.* 2019, Melzer *et al.* 2000, Menkhorst, 2008, White, 1994). Proximity to protected areas and national parks certainly appears to be linked to increased diversity in many species (Kreuzer and Huntly, 2003), as these areas provide refugia from many threatening processes (e.g. Habitat loss and fragmentation as a result of urbanisation).

In areas where these refugia are not present, such as in regions with large scale agricultural developments, available habitat is limited to remnant natural vegetation, wildlife corridors and wind breaks (Lindenmayer and Nix, 1993). The region surrounding Gunnedah is predominantly farmland and modified grazing vegetation. While all samples were within the same bioregion (IBRA), the land was heavily impacted by human activities, and as such, Gunnedah samples were consistently negatively associated with more desirable habitat traits, such as dense eucalyptus vegetation (Figure 4.7). Conversely, Port Macquarie is home to a significant koala population, as a result of the area comprising "ideal" koala habitat (Canfield, 1987). While this habitat has been disturbed by urban development since European settlement, much of the area surrounding Port Macquarie is protected as either national parks, or state forests, with habitat corridors being present throughout the region, even directly through urban areas. This may allow for more natural movement of individuals, with less barriers to dispersal. Despite this, Port Macquarie is also a major town/city, and as such contains many residential areas and fragmented habitats. Roadways are present at high frequency within this region, and intersect koala habitat more so than in other similarly populated areas (i.e. Sydney), which has a potential bias sampling towards animals who had been injured (as most of these samples were opportunistically sampled after being treated for injury or disease).

Just as national parks provide refuges for wildlife, habitats which have not been modified by human activities, or are minimally used by the public also provide areas of refuge. Despite being much closer to a major urban centre than other sample sites, the samples collected from the Sydney region were in close proximity to not only the Royal National Park (NSW), but also to a major military reserve, which is not accessible to the public. Many of the individuals collected at this site were in areas categorised as agricultural land, or modified and native vegetation, however, the proximity to protected areas means this region is arguably the most undisturbed of all sampling regions in this study. A previous study on koalas in this region suggested that this military reserve offered an important refuge for this koala population (Lee *et al.* 2010b). Overall, less genetic variability between individuals was observed within this region, which is consistent with the presence of large-scale habitat modification and urbanisation in the broader Sydney region. Interestingly, this region also contains some of the highest levels of genetic diversity in Australia (refer to Chapter 3). One of the major factors which consistently explained variations in genetic structure within and between regions was the quality and continuity of favourable habitat. Major highways and towns appear to act as partial barriers to gene flow, as animals may not be able to move through/across areas of unfavourable habitat, and higher mortality rates in these areas may act as an influencing factor (Gordon *et al.* 1990). However, in areas where favourable habitat is abundant and undisturbed, animals may not need to migrate as far to find appropriate resources, so these regions are likely to show less variation locally, but more distinct patterns of structuring over larger areas. These patterns did not appear to be consistent between all regions sampled in this study however, and so future management plans should carefully consider these effects between regions. In this study,

genetic structure of koala populations from both Lismore and Sydney were influenced by both geographic distance, and environmental factors. However, geographic distance between samples did not affect the structure of populations from Gunnedah and Port Macquarie, and other factors including variation in rainfall and proximity to a national park (or protected area) better explained genetic patterns.

Conclusions and recommendations

Dispersal can be influenced by both biotic and abiotic factors, which in turn affects the genetic structuring of populations at both a small and large scale. Understanding these drivers is important as it provides tangible cornerstones for conservation management. While it is generally accepted that habitat type can influence populations, particularly in dietary specialists such as the koala, few studies have investigated this relationship. Genetic differentiation between, or adaptation to habitat types has been suggested in previous studies at a larger scale (Johnson *et al.* 2018, Kjeldsen *et al.* 2019, Kjeldsen *et al.* 2016), but has not been suggested at such a small scale to date. Habitat structure and environmental variables are expected to affect genetic sub-structuring, and although direct geographic distance did not appear to be a significant factor to explain structuring patterns in all regions, variability in habitat structure does appear to significantly affect gene flow on a small spatial scale. Clear differences were observed between habitat types, even if they occurred within the same geographic region. This result is a clear indicator that habitat structure and other environmental factors should be taken into consideration when management plans are devised for koalas across Australia.

Chapter 5: Parentage Testing and Traceability

Development of high-utility genomic resources for ongoing evaluation of captive and wild koala (*Phascolarctos cinereus*) populations for parentage, traceability and diversity

Research Objectives and Chapter Summary

Background/Gap in knowledge

Conservation and captive breeding programs aim to ensure the continued long term survival of a species. Successful conservation relies on maintaining a maximum level of genetic diversity, and avoiding increases in inbreeding. Genetic-based pedigrees and diversity estimates are more accurate than more traditional methods based on breeding records alone. With the reducing costs of DNA sequencing technologies, it is now more feasible to create low density, genome-wide panels of SNPs for wild species. The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial which, despite having a specialized diet, is able to thrive in a wide variety of environments and is found in zoos around the world. Here, I have identified a panel of 1,850 SNP markers, which are informative to assess diversity, assign parentage, and determine the source population of an individual koala across the entire species range.

Aims

i) Develop a panel of SNP markers to be used in testing for parentage, traceability, and routine diversity studies,

ii) Assess and evaluate the usefulness of these markers when used across the species range,

ii) Assess the usefulness of these markers in a captive breeding population.

Significance/Conclusions

In this study, I have identified 1,850 candidate SNP markers in koalas, which are informative to assess genomic diversity, can accurately assign parentage, and can assign individuals to wild source populations from across the species range.

Here, multiple pedigree errors were detected when comparing breeding records with genetic data (initially using up to 2,015 genome wide SNPs) in the captive breeding population. This highlights the need for additional methods of recording parentage in captive koala populations, so that management of breeding plans are well informed.

Abstract

Conservation and captive breeding programs aim to ensure the continued long-term survival of a species. Successful breeding programs rely on maintaining the optimum level of genetic diversity, whilst minimising inbreeding. Traditionally, this is performed through animal pairing records, although the accuracy of this method can be limiting due to non-observed external mate contributions. Genomic pedigrees and diversity estimates are far more accurate than traditional methods based on breeding records alone, as these do not rely on observational data. With the costs of DNA sequencing and routine genotyping technologies reducing, it is now more feasible to create low-density (e.g. ~1,000s of SNPs), genome-wide panels of SNPs for routine population genetic analysis of wild species. The koala (*Phascolarctos cinereus*) thrives in a wide variety of environments across Australia, and is also found in zoos around the world. Yet no common genomic tools exist which can profile parentage, traceability and diversity, for both captive and wild populations. Here, I have identified a panel of 1,850 SNP markers (split across two distinct panels), which are highly informative to estimate assign parentage, determine the source population of an individual koala across the entire species range (based on n=850 SNPs), and estimate species-wide diversity (based on n=1,000 SNPs), and can be used as an international reference panel for ongoing data collection.

Introduction

Successful conservation programs aim to ensure the continued long-term survival of a species in potentially changing environments. One of the most effective ways of achieving this is by maintaining an optimum level of genetic diversity, and avoiding increases in inbreeding (Frankham, 2008). Genetic diversity increases the likelihood that a species will be able to adapt to changes in their environment. In a captive breeding setting, particularly if the population is part of a reintroduction program, maintaining genetic diversity is important because individuals who are successful in captive settings may not be well suited to wild environments (Frankham, 2008). As such, a high level of captive genetic diversity ensures that the ability to adapt in other environments is not lost.

The loss of diversity can lead to a reduced ability for a species to adapt, similarly, it is also important to avoid mixing individuals from highly divergent populations, as this can potentially result in outbreeding depression which can also become problematic in maintaining fitness. When breeding individuals who are highly divergent, their offspring may exhibit lower overall fitness than the parents (Frankham *et al.* 2011). This can arise when the parent individuals are adapted to very different environments, and the offspring display a transitional phenotype which is not well suited to either parental environment. This can be a common problem in captive breeding situations, as animals are often not sourced from the same population or region, and in extreme situations without prior knowledge, cryptic sub-species may be housed together, resulting in hybrid offspring (Trigo *et al.* 2013). Similarly, in management plans where the ultimate goal is to reintroduce animals to the wild, it is important to understand if the animal is suited to the new location, to increase the likelihood of survival (Frankham, 1995, Gilligan and Frankham, 2003). The ability to accurately assess genetic diversity and relatedness between individuals and/or populations is essential to any conservation program. Traditionally this has been achieved through morphological classification, and paper pedigrees. However, both of these methods often have high error rates (Visscher *et al.* 2002), particularly when animals are housed in large groups and are polygamous, and where cryptic species may be present (Reid *et al.* 2014). More recently, genetic techniques have been employed to improve the accuracy of traceability and pedigrees in conservation programs (Allendorf *et al.* 2010).

Genetic resources are a useful tool for informing management decisions. However, if these resources are to be informative across spatial and temporal scales, the resource needs to be universally applicable and widely accessible. Accurate estimates of diversity (including heterozygosity, allele frequency distributions and average relatedness), provenance (population traceability), and individual relatedness (parentage assignment), are required for comprehensive management of any species (Frankham *et al.* 2019). These resources are often utilised in model organisms, or organisms of commercial importance, but are rarely available for non-model organisms (Guppy *et al.* 2018). Studies in non-model organisms are often conducted at a small scale, resources are developed multiple times, and are not directly

comparable (Frankham *et al.* 2019). This limits their utility at a species-wide scale, and for ongoing monitoring and management.

Similar to *in situ* management, for captive breeding programs to be effective, they must strike a balance between maintaining diversity, and simultaneously avoiding mixing animals from populations which are too divergent (Frankham, 1995, Frankham, 2008, Frankham *et al.* 2011). In a captive breeding setting this can be challenging because founder individuals can be limited, and even if it is possible to source individuals from many different regions, it is often difficult to house large numbers of breeding animals. While many captive breeding populations are now managed by an exchange of animals between zoos and wildlife parks, the founders for these captive populations were originally sourced from wild populations. In many cases, these are well documented, but there are often situations where an animal may be misclassified. These misclassifications may occur at a species level, leading to species hybridization (or a complete lack of breeding), or at an intraspecific level (population/regional), which may lead to outbreeding depression (Houde *et al.* 2011). This is particularly true when a wild animal has been illegally transported, resulting in little to no paper records of the animal's pedigree or origin. Morphological assessments are often conducted to classify animals which come into captivity from the wild, but classifying species based on morphology alone is prone to error (Zeh and Zeh, 1994).

Microsatellite and RAPD markers have been developed to assess genetic variation and parentage within captive breeding populations, however these are not widely utilised (Ruiz-Rodriguez *et al.* 2014). These markers are also not always informative for tracing captive animals to their source populations. This is partly because there has been a lack of species wide sampling distributions to compare results to at the time of marker development (Kjeldsen et al. 2019). Employing a combination of morphological classification, with molecular screening techniques allow for a much more accurate classification. Genomic markers such as genome-wide SNPs produced by single multiplex assays (i.e. GBS or SNP arrays) provide a cost-effective alternative to traditional markers (Guppy *et al.* 2018), and large numbers of markers can be developed quickly, and in the absence of other genomic resources. Using these markers, levels of baseline relatedness and diversity can be established, and monitored over time, leading to more effective management of breeding, and conservation programs.

The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial which has historically responded well to breeding in captivity. Despite having a specialised diet, they are able to thrive in a wide variety of environments and are found in zoos around the world. In the wild, koalas are distributed from the tropics in far north Queensland, through to the temperate regions in South Australia. The koala is listed as vulnerable under the IUCN red list, and due to its iconic status, is often targeted as a key species for conservation (Melzer *et al.* 2000). Many captive breeding populations exist within Australia, and around the world, however many of these populations rely on paper pedigrees to inform breeding pairs/groups, if this is taken into consideration at all. With the recent development of genome-wide SNP datasets

(Kjeldsen *et al.* 2019), and the sequencing and assembly of the koala genome (Johnson *et al.* 2018), developed based on individuals spanning the entire species distribution, there is now a valuable opportunity to develop a cost effective, versatile genome-wide marker panel which can be used to not only accurately calculate parentage, but also to trace captive individuals to source populations (region). This will ultimately allow for more informed breeding programs, and could offer a cost effective option to inform active management/translocations of individuals in the wild.

This chapter aims to provide a comprehensive genomic resource for management of the koala, which can be utilised in the ongoing monitoring of wild and captive populations across the species range. I aim to develop and evaluate a set of genome-wide SNP markers based on a comprehensive genomic survey, which are informative to trace an individual koala back to its source population, while also being informative for parentage determination. These markers could potentially be used to assess relatedness in captive populations, and could also be useful as a diagnostic tool where active management of wild populations is required.

Methods

Samples and initial filtering

All genotypes used in this study were produced using the Diversity Arrays platform (DArTseq™), and initially filtered as per Kjeldsen *et al* (2019). Briefly, to ensure markers were present across all populations (including captive populations), any individuals with a genotyping call rate <80% were removed from the dataset, and any SNP with MAF <0.02 were also removed to avoid analysing monomorphic loci and low frequency sequencing errors. Informative markers were selected for identity analysis, parentage analysis, population traceability, and diversity metrics. Initially, the complete DArTseq dataset was filtered using dartqc v2.0 (available at: https://github.com/esteinig/dartQC), to remove markers with low read depth (--read_counts <7 reads), markers which were duplicated based on sequence similarity (--cluster >0.95), had poor repeatability (--RepAv <0.95) and which displayed skewed heterozygote calls based on read count ratios.

Marker selection: Parentage

To estimate the number of markers required to accurately assign parentage and identity, probability of parentage exclusion were calculated in Genalex (Peakall and Smouse, 2006) using the full SNP dataset, and all individuals. To ensure SNP markers would be informative across the species range, samples spanning the entire species distribution, including both natural and introduced populations, were used in this study (Table 5.1). A captive breeding population from San Diego Zoo, with information on parent/offspring groupings, was also used to test how informative the SNPs are on a captive population with a recorded pedigree. San Diego Zoo is responsible for many conservation breeding programs, and its koala conservation program is the largest in the world. San Diego Zoo is the main source population for other zoological parks outside of Australia, so it is important that any SNP markers used for testing of captive populations are informative for this captive group.

Following initial filtering, metrics including call rate, MAF, heterozygosity (expected and observed), F_{ST} (per SNP) were calculated and each SNP was ranked according to these values, using custom R scripts. To select additional SNPs to be used for parentage assignment, only SNPs that displayed a call rate of 100%, and average MAF >0.2 were retained.

Marker selection: Individual or population provenance

In order to differentiate individuals or groups, diagnostic SNP markers ideally display high average MAF values (MAF >0.2) across and within populations. All SNPs were assessed for average MAF across all populations, to ensure each candidate SNP had a high average MAF across all test populations.

In addition to this, to select a subset for use in population traceability, all SNPs were ranked manually according to pairwise F_{ST} values, and those displaying the highest discriminatory power (highest F_{ST} values), were retained for further analysis. To provide more resolution to population traceability, private

and rare alleles were also identified within each population (Peakall and Smouse, 2006). Markers which had a high frequency within one population, but were rare, or absent from all other populations were also retained as diagnostic markers.

Validation and Testing: Parentage and Providence

As a result of selection criteria being similar for both parentage assignment and population traceability (i.e. high MAF and discriminatory power), many loci were common between the two datasets. Following manual identification of subsets of SNPs for parentage and provenance, these subsets were merged into a single file, with any duplicate SNPs (appearing in both subsets), being merged. To ensure that these SNPs fell at unique regions of the genome, surrounding sequences of each marker identified here were aligned to the koala genome to ensure no duplicate markers were included (Supplementary Table 5.1).

In order to test the effectiveness of a combined parentage and provenance subset of markers, simulated F1 generation genotypes (n=5,000) were produced by crossing individuals from each of the sampled populations (Table 5.1), based on observed allele frequency distributions both across the range, and within each population, using the simulation function in MyKiss (Kalinowski, 2010). For population traceability tests, an additional 100 offspring were simulated for each population separately (2,100 offspring in total), to ensure that simulated offspring were not produced by crossing between populations. All simulations were produced with combinations of known parental contributions, and a subset of 10% of offspring with an unknown parent. These datasets were simulated with an average of 10% genotyping error, and 5% of missing data per SNP. Simulated datasets were then used to test candidate SNPs for effectiveness of parentage assignment, and population traceability.

Higher levels of inbreeding are expected in captive breeding populations, and this can result in incorrect parentage assignments if the parents are closely related. An additional subset of SNPs, which were particularly informative in the San Diego Zoo population, was selected for testing (MAF >0.2, present and polymorphic across all individuals in the full data set). These markers were also incorporated into the combined parentage and provenance subset.

To ensure that the recorded pedigree was correct, a full set of 2,015 SNPs which were polymorphic in the San Diego Zoo population were used to create a genomic relationship matrix (GRM) in PLINKv1.9 (Purcell *et al.* 2007), using the –make-rel flag. Where pedigree errors were identified, the pedigree was amended to reflect the most likely parents wherever possible. If it was not possible to reconstruct parent-offspring groups with the available data, family groupings were inferred using Colony (described below) and the GRM.

After candidate SNP markers were identified for use across all populations (n=850), each subset was used to assign parents to simulated offspring using the programs Cervus and Colony (Jones and Wang, 2010, Kalinowski *et al.* 2007) to test the utility of each subset. For each test, initial simulations were

run to assess 5,000 simulated offspring, with mating system defined as polygamous with inbreeding (simulated at 1% per generation), in a species with a diploid genetic framework. For all simulations, a minimum of 50 typed loci were required, with a maximum of 25% unsampled parents and a genotyping error of up to 10%. Following simulations, all simulated F1 progeny were assigned back to both parents, using an average LOD score approach. A separate assignment run was performed including recorded parent-offspring groups using empirical data from individuals sourced from San Diego Zoo to test accuracy of assignment within a real breeding population, with a recorded pedigree.

To test that candidate SNPs were highly differentiated between natural populations and regions, a discriminant analysis for principal components was calculated and visualised using the DAPC function in the R package *adegenet*. This was to ensure that the candidate SNPs would be useful to determine the region individuals were sourced from. Individual/population assignment tests were performed based on each subset of SNPs, to assess reliability of assignment to source populations from across the species distribution. Assignment tests were calculated using a Bayesian approach as described in Baudouin and Lebrun (2001), in GeneClass2 (Piry *et al.* 2004). In addition to assigning all wild individuals, the San Diego Zoo population was used as a test captive population, because founder individuals have recorded wild source populations.

Table 5.1. Parentage assignment for simulated datasets, simulated F1 generation based on randomly crossing individuals from each population. Simulated datasets contain up to 10% genotyping error and up to 5% missing data.

Population	n [simulated]	Proportion correctly assigned both parents (simulated F1 only)	
		Parentage and Provenance Subset	Entire Dataset (unfiltered)
Wild Animals	5,400	1.0	0.98
Magnetic Island (MI)	100	0.99	0.98
St Bees Island (SB)	100	1.0	0.99
St Lawrence (SL)	100	1.0	0.98
Maryborough (M)	100	1.0	0.98
Moreton Bay (MB)*	100	1.0	0.98
Koala Coast (KC)	100	1.0	0.98
Ipswich (I)	100	1.0	0.99
Lismore (LI)	100	1.0	0.99
Woolgoolga (W)*	-	-	-
Gunnedah (GD)	100	1.0	0.99
Port Macquarie (PM)	100	1.0	0.99
Blue Mountains (BM)	100	1.0	0.99
Campbelltown (CT)	100	1.0	0.99
Southern Highlands (SH)	100	1.0	0.99
South Gippsland (SG)	100	1.0	0.97
Strzelecki (SZ)	100	1.0	0.97
French Island (FI)	100	1.0	0.97
Cape Otway (CO)	100	1.0	0.97
Hamilton (H)*	-	-	-
Mt Lofty (ML)	100	1.0	0.97
Kangaroo Island (KI)*	-	-	-
San Diego Zoo (SDZ)	100	1.0	0.97

Marker selection and validation: Diversity

A second subset of SNP loci was selected specifically for estimation of diversity metrics. This was separate to the initial panel selected for parentage and providence, as any stringent filtering would have skewed allele frequency distributions, leading to inaccurate or biased diversity estimates. This panel was selected (following initial filtering), by randomly selecting a set of markers which matched the allele frequency distribution of the original, full dataset. Correlations between the entire dataset, and the selected subset were calculated to ensure that key diversity estimates, such as heterozygosity (He and Ho), were not skewed. This subset was tested and validated separately from the parentage and provenance subsets as diversity metrics required an unbiased sampling strategy, which accurately reflected the population-level metrics.

Results

Marker selection: Parentage and Population Traceability

To test how many SNPs were likely to be needed for accurate individual assignment, probability of identity, and probability of exclusion plots were calculated for each population using all SNPs (Figure 5.1). These indicated that, in an outbred population, beyond a combination of 150 randomly selected SNPs, there was little improvement in discriminatory power for value for additional SNPs selected using both an F_{ST} and MAF based approach (p=1.79E-4).While as few as 150 markers were informative for individual assignment, larger numbers of markers were tested to account for scenarios of low variation between individuals, or high levels of inbreeding.

For accurate diversity estimates, a greater number of markers was required compared to what was needed for individual discrimination. A subset of 1,000 SNP markers for each subset (two distinct groups), was selected for further testing. The first (parentage) was selected based on high average MAF (>0.32), and the second (provenance) also included high average pairwise F_{ST} values (>0.41), private and rare alleles to help differentiate populations across Australia. Once combined, and duplicated markers were removed, a final subset of 850 SNP markers was carried forward for testing.

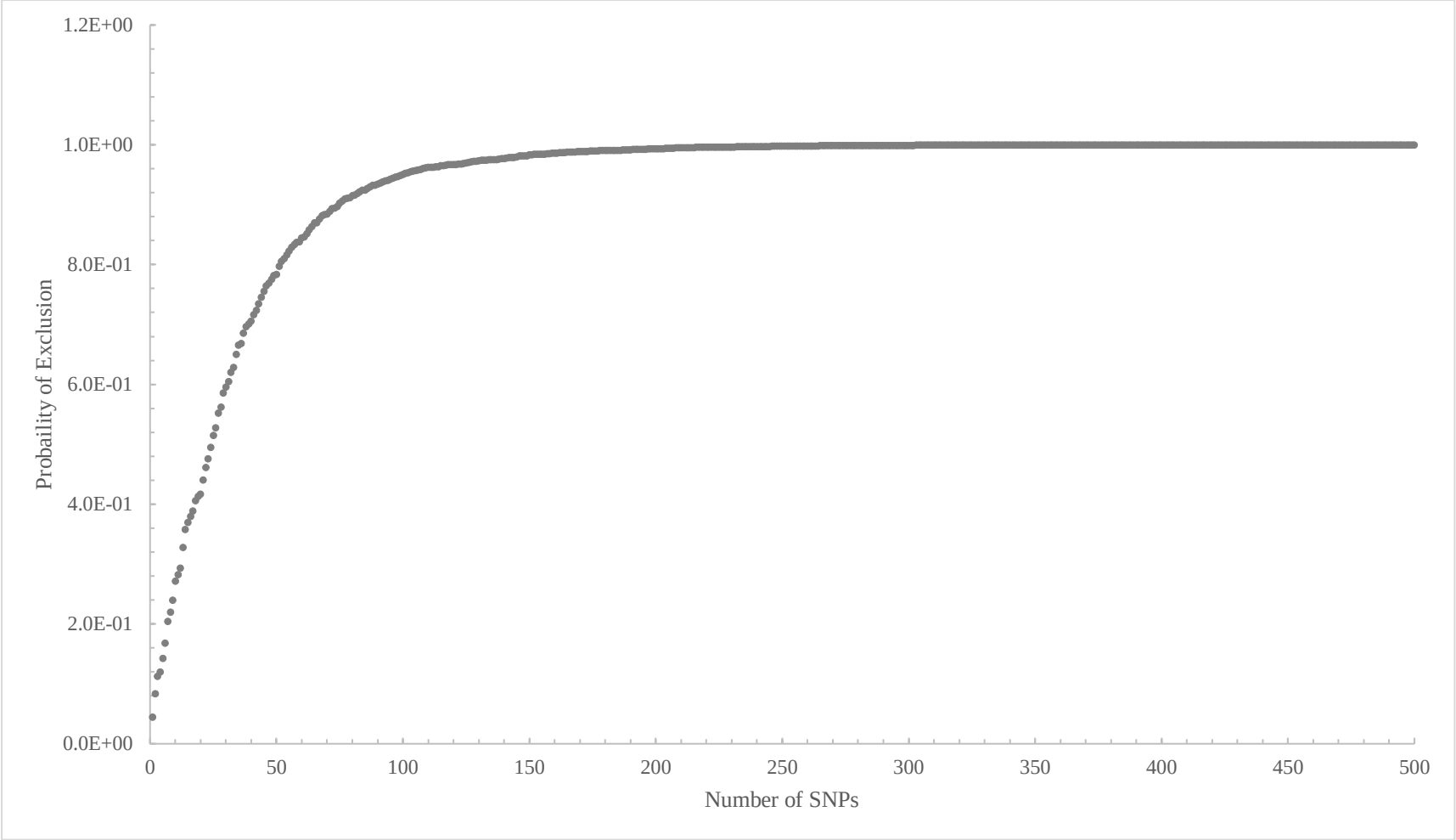
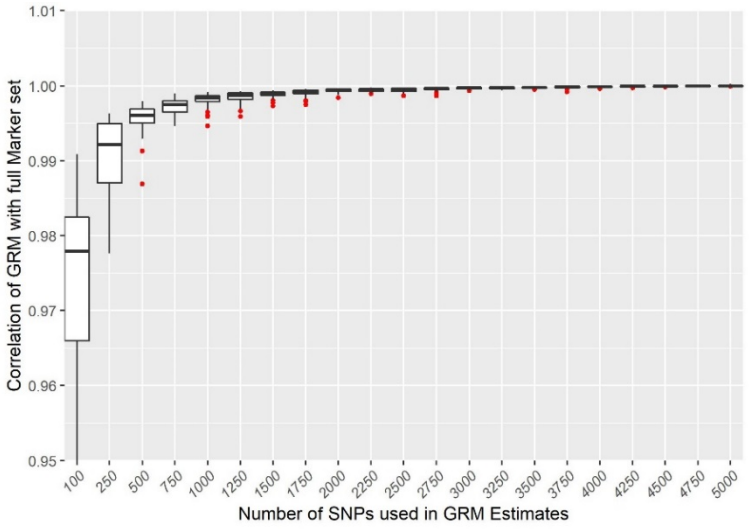
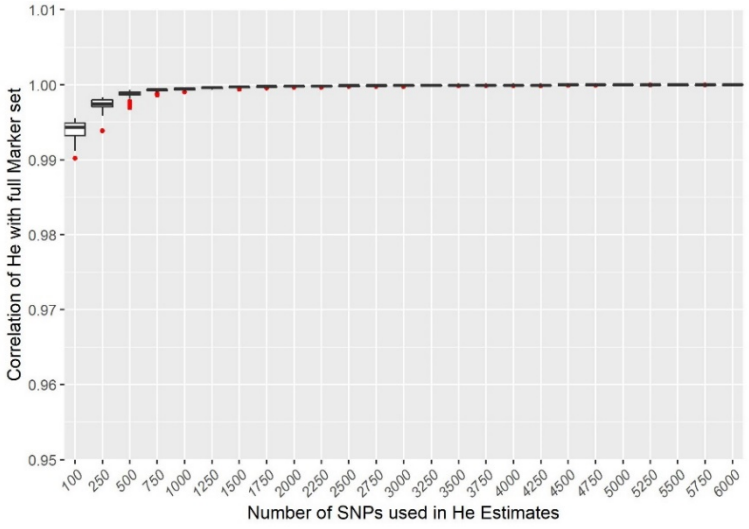


Figure 5.1. Probability of exclusion (based on 100 replicates and all markers), produced in Genalex (Peakall and Smouse, 2006). Up to 500 SNPs are shown here to avoid redundancy.

A



B



C

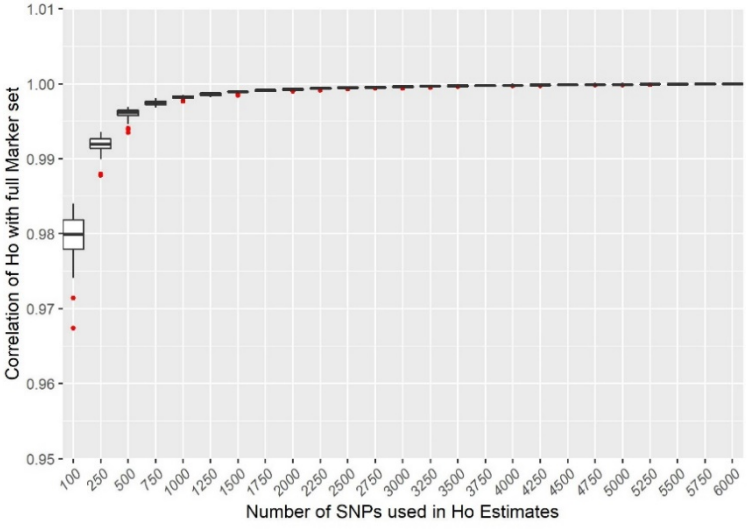


Figure 5. 3. a, b and c. Power analysis – Correlations between random subsets of markers and the full dataset as described in Kjeldsen *et al.* 2019 Parentage and Provenance Subset – R^2= 0.98±0.015, Diversity Subset – R^2=0.99±0.005

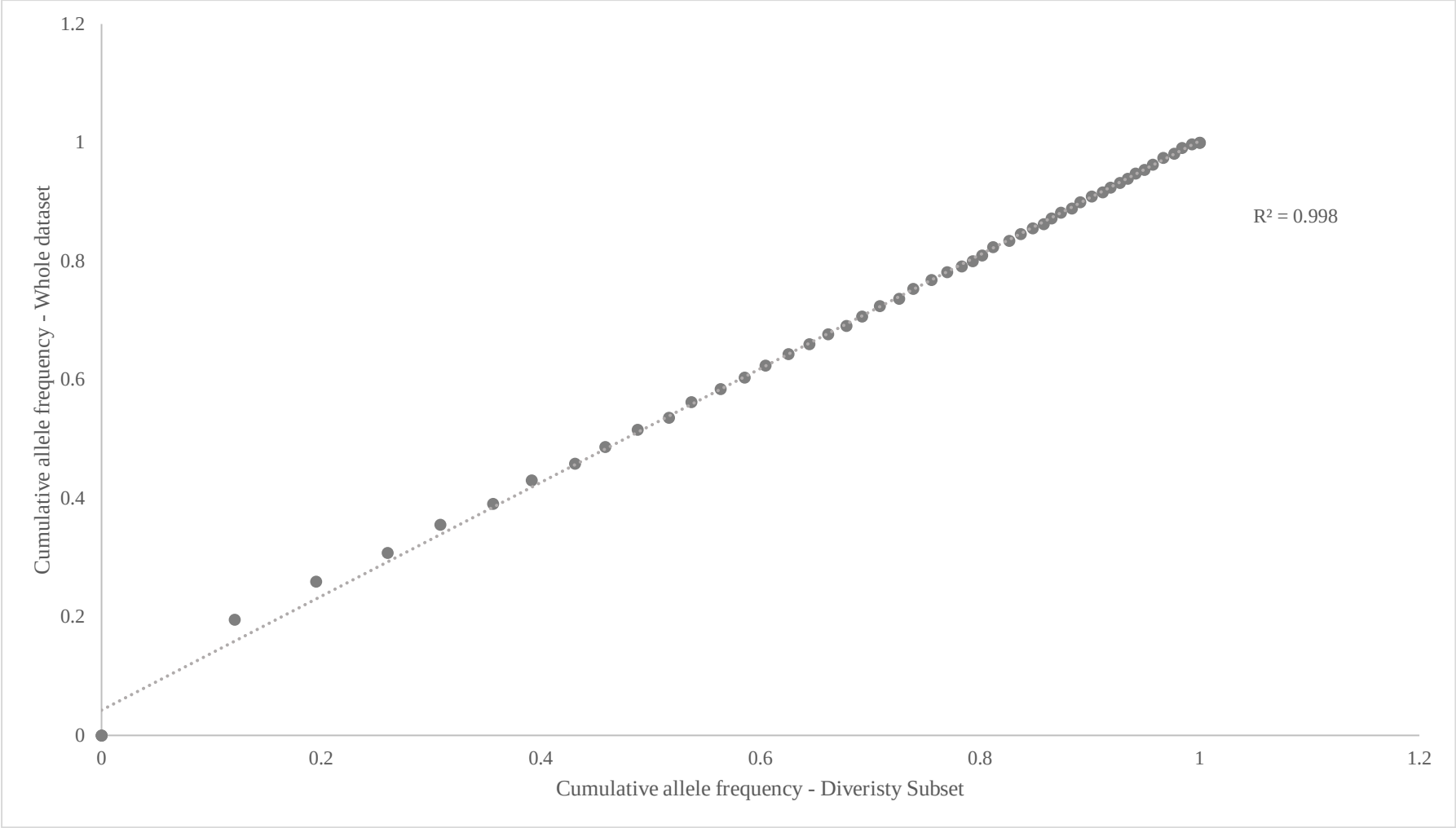


Figure 5.4. Comparison of allele frequency distribution of whole dataset (>5,000 SNPs), and the diversity subset (1,000 SNPs)

Testing and Validation: Pedigree Accuracy and family relatedness

To assess the accuracy of the recorded pedigree, a GRM was calculated between all genotyped individuals using 2,015 SNPs with high call rate (>0.9) and high MAF (>0.1) within the San Diego Zoo population. Relatedness levels revealed multiple inconsistencies within the recorded pedigree. Only 30% of recorded pedigree assignments could be corroborated with the full genomic dataset. As it was not possible to definitively correct all pedigree errors due to the possibility that true parents were not available to be genotyped, family groupings were inferred based on results of the GRM relatedness matrix, combined with Colony results (Supplementary Table. 5.2). These family groupings were then compared to subsets of candidate SNPs.

Subsets of SNPs selected for parentage assignment returned highly accurate assignment of both parents in wild simulated populations (Table 5.2). Assignment rate for simulated offspring was 100%, based on a subset of 850 SNPs within the parentage and provenance selection. No mis-assignments were identified in the simulated datasets. Due to incomplete genotyping of animals (and potential parents) within the captive population tested, a lower assignment rate was expected, and only 28% of individuals were assigned to at least one parent as recorded within the original paper pedigree. However, when parentage assignment was compared to the corrected pedigree (based on the whole dataset Colony results) in Supplementary Table. 5.2, the parentage and provenance subset correctly assigned >99% of offspring to at least one parent.

Table 5.2. Parentage assignment for empirical datasets from individuals from San Diego Zoo.

	n	Proportion Correctly Assigned (original paper pedigree)	Proportion Correctly Assigned (corrected genomic pedigree)
Both Parents Genotyped	8	0.5	0.97
Single Parent Genotyped	24	0.21	0.97
Parents Unknown	6	-	-

Proportion of correctly assigned populations

Both subsets of SNPs (those selected for parentage and provenance, with and without the inclusion of rare alleles) were able to accurately place samples to their correct source population, with the addition of rare and private alleles resolving population groupings marginally more than those selected based on MAF and F_{ST} alone (Table 5.3). Results of DAPC analyses displayed that the combined parentage and provenance markers provided higher resolution and accuracy for population assignment than other SNPs (Supplementary Figure 5.1). Without rare and private alleles, 86.9% of individuals were correctly assigned to their source population. A large proportion of mis-assignments were attributed to individuals from southern regions, with 49.4% of individuals from Victorian and South Australian populations being assigned to French Island, and only 51.4% of individuals being assigned correctly to their source population. The majority of samples from South Gippsland and Strzelecki were assigned correctly (94.1%), with only a single individual from each region being assigned to a neighbouring population. For all remaining populations tested, 93.8% of individuals were correctly assigned to their source population, with a further 4.7% being assigned to a neighbouring population. Only 3.6% of individuals were mis-assigned to populations or regions which were unlikely to be their source population. However, when assignments were based on discrete genomic clustering patterns (based on NetView results – Chapter 3), assignment rate improved to 100% across all individuals (Table 5.3).

Population assignments based on the larger panel of SNPs (the full parentage and provenance subset; 850 SNPs) yielded more accurate results, with 94.5% of individuals being correctly assigned to their source population. As with the panel selected using only MAF and F_{ST} filtering, when Victorian and South Australian populations were considered alone, assignment rate was lower and dropped to 90.4%, with only a marginal improvement in assignment of individuals from New South Wales and Queensland (+0.6%).

Table 5.3. Proportion of individuals correctly assigned to a source population, based on 100 simulated individuals per population. Assignments were based on a naïve Bayes classifier approach in the R package *assignPop* (Chen *et al.* 2018)

Population	Proportion correctly assigned to source population	Proportion correctly assigned region (based on NetView clustering)
Magnetic Island (MI)	1.00	1.00
St Bees Island (SB)	1.00	1.00
St Lawrence (SL)	1.00	1.00
Maryborough (M)	1.00	1.00
Moreton Bay (MB)*	-	-
Koala Coast (KC)	1.00	1.00
Ipswich (I)	1.00	1.00
Lismore (LI)	1.00	1.00
Woolgoolga (W)*	-	-
Gunnedah (GD)	1.00	1.00
Port Macquarie (PM)	1.00	1.00
Blue Mountains (BM)	1.00	1.00
Campbelltown (CT)	1.00	1.00
Southern Highlands (SH)	1.00	1.00
South Gippsland (SG)	1.00	1.00
Strzelecki (SZ)	0.75	1.00
French Island (FI)	0.65	1.00
Cape Otway (CO)	0.10	1.00
Hamilton (H)*	-	-
Mt Lofty (ML)	0.37	1.00
Kangaroo Island (KI)*	-	-
San Diego Zoo (SDZ)	0.97	1.00

*excluded from simulated F1 analysis due to low actual sample size, resulting in unreliable allele frequency estimates

Marker selection and validation: Diversity

Diversity estimates required higher numbers of SNPs to provide an accurate reflection of common diversity metrics (e.g. heterozygosity, and allele frequency distributions). Up to 1,000 markers were required to provide accurate results for diversity metrics (R^2>0.99, Figure 5.3a,b,c, Figure 5.4). The final panel selected for diversity assessment accurately reflected the allele frequency distribution of the original dataset, which includes samples from populations across the species range.

Discussion

Successful conservation and management of species requires an integrated and consistent approach across temporal and spatial scales. This requires resources which have been developed based on a comprehensive species-wide survey, and so are informative across the species range. This resource should also have broad utility for conservation questions, and should be widely accessible. Here, I present for the first time a comprehensive genomic resource for koala management, which may be used as a foundation for ongoing management of this species. In this study, I have identified 1,850 candidate SNP markers in koalas, which can accurately assign parentage, assign individuals to wild source populations (n=850), and estimate genetic diversity across the species range (n=1,000).

Parentage assignment and inbreeding assessment

Previously, parentage assignment in koalas was conducted using pedigrees and breeding records, or infrequently, using microsatellite or RAPD markers (Fowler *et al.* 1998, Cocciolone and Timms, 1992, Wedrowicz *et al.* 2017). While breeding records are often more cost effective than genetic methods of parentage analysis, they are frequently prone to error, particularly in a polygamous species. Here, multiple errors were detected in the historic paper pedigree (Supplementary Table 5.3) when comparing breeding records with genetic data (initially using up to 2,015 genome wide SNPs). Only 30% of pedigree records were supported by genomic data. This highlights the need for additional methods of recording parentage in captive koala populations, so that management of breeding plans are well informed.

Accurately identifying family groups in captive koalas can be difficult as they are often housed in large, freely interbreeding groups. Koalas are typically polygamous, so if multiple males are housed with a female, it can be difficult to accurately predict paternity without genetic screening. Similarly, if offspring are not tagged/microchipped while in the pouch, cases of "joey swapping/dumping" have been observed, which makes assigning maternity equally difficult. Likewise, even if individuals are paired until mating occurs, large variations in gestation have been observed (Gifford *et al.* 2002, Reid *et al.* 2014), and the possibility of embryonic diapause may lead to errors in pedigree. Pedigree records may not account for these oversights, so routine genetic testing using informative SNP markers will allow for more precise records to be kept, and for more accurate estimates of inbreeding. Given the unknown inbreeding status of the recorded founding individuals for this population, the similarity in expected and observed inbreeding values indicates that the founding individuals were unlikely to be closely related.

When comparing numbers of markers used for parentage assignment, probability of exclusion analysis (Figure 5.2) indicated that only ~150 SNPs would be needed to accurately and consistently assign parentage. However, given the genetic variation present across the species range (Kjeldsen *et al.* 2018), and the potential levels of inbreeding in captive populations, more markers were selected to provide

redundancy and ensure long-term utility across all populations. The inclusion of markers with high call rate and high MAF, along with markers which were specific to each region, increased accuracy of both parentage and population assignments.

This increased accuracy was particularly relevant where only a single parent was known. A total panel of 850 SNPs was chosen to ensure that some redundancy was accounted for, so that if some SNPs were to show a monomorphic pattern in a small subset of individuals, others would still be informative. In real captive breeding populations, and when testing wild individuals, it is also likely that at least one parent will be unknown or untested, so an increased number of SNPs used for parentage assignment was chosen to overcome these potential deficits in data. In scenarios where all potential parents have been screened, fewer SNPs may be used to achieve the same level of accuracy and precision.

Accuracy of parentage assignment may become more difficult in populations where higher inbreeding is observed (Reid *et al.* 2014). Parent-offspring pairs, or full siblings are expected to share approximately 50% of their genomes, with half siblings sharing approximately 25%. However, the proportion of the genome shared increases if the parents are related. If this increased relatedness is not accounted for when calculating parentage assignments, it can lead to inaccurate assignment of parents or siblings. Using more markers can increase resolution, and simulations where inbreeding between half siblings was present, were still able to be accurately assigned parents with the subset of 850 SNPs identified here (Table 5.2).

Population assignment and reduced resolution of assignment to southern koala populations

Identifying SNPs for accurate population assignment is highly reliant on the ability to sample an adequate number of individuals from each population, so that accurate allele frequencies can be estimated. Enough of the natural range also needs to be sampled so that the SNPs used for traceability have enough discriminatory power to place an individual back to a wild population anywhere within the natural range. If only a small area of the natural species range is sampled, individuals may be mis-assigned. This occurs as a small sample size may have a "bottlenecking" effect, where the allele frequencies of the sampled population do not reflect that of the original population (Luikart *et al.* 1998, Watterson, 1984).

This study utilised samples spanning much of the koala's natural and introduced range in Australia, encompassing both the northern and southern regions, which have been shown to be genetically divergent (Kjeldsen *et al.* 2019). The SNPs identified here were able to successfully place individuals (both real and simulated), back to a source population at a regional level (or discrete genetic cluster), with variable degrees of resolution. Ecological history can also affect population assignment, and populations which have a history of genetic bottlenecks, translocations, or which have high rates of gene flow, may result in highly admixed populations. This admixture can reduce the resolution of population assignment, leading to individuals being assigned only to a relatively large geographic

region, rather than at a finer population scale (Neethling *et al.* 2008). This trend was observed when attempting to assign individuals from mainland Victoria and South Australia back to their source populations. Up to 42.7% of individuals from the southern populations were assigned to the French Island population. Several subsets of SNPs were tested to attempt to resolve this, however no SNPs were able to be identified to accurately differentiate these populations. This is likely a result of the several translocations which have occurred between French Island, and mainland Victoria throughout the 20th century (Melzer *et al.* 2000, Menkhorst, 2008). Including markers which were private to these populations, or locally common, but regionally rare helped to resolve some of these mis-assignments, however the rate of mis-assignment in southern populations was still higher than in other regions. This difficulty in separating southern populations is reflected in the results presented in Kjeldsen *et al.* 2019, which showed very little variation within this region. Southern koala populations have a history of multiple reintroductions, following significant population crashes in the late 19th to early 20th Century. French Island (which is itself an introduced population, founded by as few as 20 individuals from mainland Victoria) was the source for many reintroductions. Many of these reintroductions occurred in the first half of the 20th Century, and given that only approximately ten generations separate extant southern populations from originally introduced populations, it is unlikely that populations have differentiated sufficiently to allow for fine resolution population assignment. Exceptions to this trend were individuals from the South Gippsland and Strzelecki regions, which were genetically distinct from all other extant Victorian and South Australian populations sampled, and were able to be correctly assigned to a single overarching population within the South Gippsland region. This is consistent with patterns shown in previous studies using microsatellite markers (Neaves *et al.* 2016; Wedrowicz *et al.* 2018). Prior to the inclusion of population specific rare alleles to aid in population assignment, for all other groups throughout Australia, up to 91.7% of individuals were assigned to their source population, with 4.7% of the remaining individuals being assigned to a neighbouring population, leaving only 3.6% of individuals mis-assigned. Where individuals were assigned to neighbouring populations, this was largely in areas which have been suggested to have higher rates of gene flow, such as in the south-east Queensland region (SEQLD), and within the Sydney Basin region (Kjeldsen *et al.* 2019). Given that the overall rate of mis-assignments did not improve with a larger dataset (i.e. the full SNP dataset), this indicates that an increased panel would be unlikely to further resolve this. When the captive population from San Diego Zoo was assigned to wild source populations, all but three individuals were assigned to the Ipswich population, within the SEQLD region. This finding is in keeping with zoo records, which indicate that a large proportion of the captive breeding population was sourced from the SEQLD region. Considering the importance of the San Diego Zoo population to conservation of the species as a whole, the fact that only a small proportion of overall species diversity is captured in this population is concerning. If captive breeding populations are to be of use to wild conservation efforts, these populations should aim to be representative of the entire species range, and to capture levels of genomic variation as close to wild populations as possible. It is generally impractical to assume that a single

captive population would allow for the preservation of species-wide diversity, and so a more realistic suggestion might be a collaborative effort between institutions, where each institution is responsible for maintaining a particular genetic sub-group of koalas. Koala populations from the Sydney basin region have previously been identified as a "reservoir" for species diversity (Kjeldsen *et al.* 2019), and so including individuals from this region in future breeding plans may introduce more variation to captive "insurance" populations. Likewise, with regular genetic screening of individuals, if inbreeding levels begin to increase, an informed exchange of individuals between captive populations may help to reduce loss of captive diversity.

Diversity Assessment

Unlike the parentage and provenance subset, estimates of genetic diversity required a minimum of 1,000 markers to accurately reflect allele frequencies observed in a larger genomic dataset (Figure 5.4). This is because, while selecting markers for assignment purposes relies on highly differentiated SNPs (i.e. the tail ends of the distribution), accurate diversity estimates require sub-sampling equally across the marker distribution.

Diversity assessment and tracking over time are a vital part of conservation and management. In order for these results to be comparable between regions, and over time, a standardised resource is needed. The panel of markers presented here for diversity estimates is based on the most comprehensive genomic survey to date in the koala. This subset of markers is largely unbiased, and provides estimates for allele frequencies, heterozygosity, and average genetic distance, which are highly correlated (R^2 >0.99) with estimates obtained from the larger survey. While it is still possible to utilise a larger panel of markers for this purpose, a smaller subset allows for a more cost effective approach (Guppy *et al.* 2018).

Future development of a genotyping assay

The SNPs identified here provide a useful tool to aid captive management of koalas, to assess relatedness within actively managed wild populations, and provide unique identity tracing for provenance. Importantly, the marker subsets provided here should be used separately, as they have been selected based on different criteria. The parentage and providence marker set should not be used to estimate diversity, as these markers will inflate diversity estimates due to their higher discriminatory power. Likewise, the second panel selected for estimating diversity should be used to track changes in genetic diversity over time, and can be used across the natural species range, or in captive populations. This panel may also be used for the purposes of parentage assignment and population traceability, however these markers may reduce accuracy by including a higher proportion of markers which do not adequately separate individuals. However, the practicality of applying these to conservation management is questionable without the development of an accessible, affordable and reliable genotyping method. While the development of a solid-state genotyping array may be prohibitive in

terms of monetary and time costs (Guppy *et al.* 2018), a viable option may be to develop a target-capture sequencing method for the SNP sequences provided here (Rohland and Reich, 2012). However, it is equally important for this resource (and resulting data) to be freely available to all stakeholders, so that results may be directly compared between regions, and across time points. A universal resource such as this would allow for data-driven management decisions from a local, to a species wide scale. This approach is frequently employed in agricultural species (Guppy *et al.* 2018a), but is rarely seen in non-model organisms. Genomic resources are quickly becoming easier, and cheaper to develop. While this allows for the unprecedented ability to study non-model organisms which have previously been overlooked, it has also led to duplication of studies by different research groups, due to a lack of publicly accessible databases to facilitate data sharing. This redundancy in research is not an efficient use of funding or time, and these datasets are often unable to be directly compared. The most effective approach to management of a species involves an integrated approach, involving as many stakeholders as possible (Caudron *et al.* 2012).

Chapter 6: General Discussion and Management recommendations

Successful management of a species requires a thorough understanding of its biology, and the threats it faces. Even with a baseline understanding, an integrated, cross-disciplinary approach is needed to implement an effective management strategy. Here, in partnership with research groups and wildlife organisations (see acknowledgements), I have collected the most extensive genomic sample resource to date, with many hundreds of samples spanning the natural and introduced range of the koala. Using novel genomic approaches, this sample base represents one of the most comprehensive datasets to date, to investigate phylogenetic relationships between koala populations. These results have supported the classification of koalas as a single ESU, with regional differentiation. I have uncovered genetic links between different environments, and trends in population structure across Australia, providing preliminary indications of adaptation to specific bioregions. This information will inform active management of koalas in these regions. I have also provided information on current genetic diversity of koala populations across Australia, using a universal method, meaning that all results arising from this thesis are directly comparable with each other. This provides a comprehensive baseline resource for all future research and monitoring programs for koala populations. I have developed and validated a reduced panel of markers to fulfil routine monitoring requirements for captive and wild populations, offering a cost effective alternative to traditional genetic methods. Importantly, this resource is directly linked to a comprehensive species-wide survey, so all future studies may build upon existing resources.

Continuity and comparability of research is imperative to successfully manage species in changing environments. The modern era has seen some of the largest, and most rapid changes to natural environments in recorded history (Braje and Erlandson, 2013, McDowell, 2014). Unpredictable variations in global climate, and widespread anthropogenic activities have challenged many species, leading to mass extinctions and overall loss of biodiversity (Braje and Erlandson, 2013, Gienapp *et al.* 2008). The significant loss of biodiversity throughout the Anthropocene is largely the result of drastic changes to, and loss of global habitat. In addition to widespread deforestation and urbanisation, anthropogenic driven climate change has resulted in an increase in major stochastic events. These events include higher average temperatures, and greater variability in both temperature and rainfall (Thornton *et al.* 2014). This variability leads to longer periods of drought, unpredictable major flooding events, and more extreme bushfires (wildfires). Specialist species are at particular risk of these events as there is a higher likelihood that their specialist niche will be effected (Clavel *et al.* 2011, Gilman *et al.* 2010, Thuiller *et al.* 2005, Travis, 2003).

Of particular note are the widespread bushfires which ravaged the Australian East coast in late 2019/early 2020 (Jalaludin *et al.* 2020, Sullivan, 2020). These bushfires covered much of the

distribution of the koala, and was most severe in areas harbouring important genetic diversity for many at risk species (e.g. koalas, rock wallabies) (Kjeldsen *et al.* 2019, Sullivan, 2020). While wildfires are a natural event in Australia, land management and fire regimes have changed considerably over time, and between aboriginal management, and European settlement (Petty and Bowman, 2007, Russell-Smith *et al.* 2013). Aboriginal land management involved regular and controlled back burning, which significantly changed the landscape over the last ~50,000 years. This regular burning reduced the severity of bushfires across Australia by reducing the amount of available fuel – this led to less frequent and less severe fires (Petty and Bowman, 2007), which in turn would have had a lesser impact on wildlife. Upon European colonisation of Australia, this hazard reduction burning regime changed drastically. While hazard reduction burning is still performed regularly today, it is not often as frequent or widespread, with greater than 80% of recent planned hazard reductions not being possible due to extreme weather in NSW alone (NSW Rural Fire Service, 2009). Additionally, these activities are time consuming, complicated, and politically controversial activities, where permissions must be obtained from all relevant stakeholders, meaning that optimal fuel reduction is rarely achieved (Petty and Bowman, 2007, Russell-Smith *et al.* 2013, NSW Rural Fire Service, 2009).

In combination with climate change, and longer, hotter drought periods, this allowed for a perfect situation for widespread fires to take hold in late 2019 (Vardoulakis *et al.* 2020). Much of the area burned in this event had not been closely managed in the recent past, and was not in areas in which bushfires were previously common – leading to a large amount of vegetation acting as fuel, in protected areas. While previous habitat loss (as a result of land clearing for agriculture) has been the primary threat to koala populations in the recent past, this catastrophic event not only lead to huge (suspected) losses of koala numbers, but also loss of vital remaining habitat. While vegetation is likely to recover in the middle-long term with careful management, it is likely that the habitat structure and overall biodiversity will change significantly with the potential to impact koala populations across Australia (Bennett *et al.* 2020).

The koala is an interesting case as it appears to conflict with many of the theories surrounding specialist species. The koala is a dietary specialist, in that it feeds exclusively on eucalyptus and acacia leaves (Adams‐Hosking *et al.* 2012, Martin and Handasyde, 1999, Moore, 2010). However, its main food source it not similarly specialised in its needs (although is specially adapted to recover from fire events), and as such covers a large geographic area, crossing many climate zones and bioregions (Hughes *et al.* 1996). So, if an animal is a specialist, but the specialist niche it relies on is not, is the animal a specialist or not? Is it similarly vulnerable to the pressures of other specialists? Interestingly, while the koala species is able to feed on over 120 species of eucalypts, within any particular bioregion, they will preferentially feed on fewer than 10 species, regardless of the presence of other suitable species

(Trueman *et al.* 2017, White, 1994). Has habitat fragmentation played a role in this dietary preference? Or is this particular trait the result of other factors?

Dietary preference in the koala has been suggested to be linked to differences in microbiomes between regions, rather than differences at a functional genetic level (Brice *et al.* 2019). There is also evidence to link chemical profile of trees with palatability, and other factors such as soil type, rainfall, and average annual temperature may influence this (Moore *et al.* 2010, White, 1994). Perhaps the differences in dietary preferences have been exacerbated by reduced connectivity. Research suggests that a large portion of the microbiome is passed from mother to offspring, through the ingestion of "pap". It is also evident that not all sub-adult koalas remain in their matriarchal territory (young males in particular), meaning that there is likely some tolerance to dietary preference. So, the spread of diversity in microbiomes (and subsequent ability to tolerate difference eucalypt species) is likely driven primarily by females.

Specialist species, such as the koala, are disproportionately affected by environmental changes, as they are often unable to utilise multiple environmental niches. In order to successfully conserve vulnerable species, a thorough understanding of their biology and ecology is needed. This thesis developed the necessary resources to allow the investigation of many key aspects of koala biology, through detailed genomic assessments.

Population Structure and connectivity

As a result of the widespread Australian bushfires in 2019, overall biodiversity and habitat structure in the affected areas are unlikely to mirror the pre-fire environment (Bennett *et al.* 2020). While eucalypt species are well adapted to recover from fire events, the degree and severity of the 2019 fires may result in different species becoming dominant in affected areas. This may be problematic for species, such as the koala, which rely heavily on only a few eucalypt species for essential resources. In Chapter 4, the proximity to protected areas and national parks appeared to be linked to increased genetic diversity in the koala populations sampled, as these areas provide refugia from many threatening processes (e.g. Habitat loss and fragmentation as a result of urbanisation). In areas where these refugia are not present, such as in regions with large scale agricultural developments, available habitat is limited to remnant natural vegetation, wildlife corridors and wind breaks. In addition to the loss of unique niche habitats, these processes also fragment habitat, significantly reducing connectivity between regions. Habitat fragmentation restricts movement of animals between regions, and alters the structure of populations (Fahrig, 2003, Templeton *et al.* 1990). Fragmentation can give rise to unique populations, facilitate further specialisation/adaptation to a certain environment, and foster divergence of species (Andren, 1994, Templeton *et al.* 1990). More often however, fragmentation leads to increased genetic drift, reduction in population (genetic) diversity, and increased vulnerability to stochastic events and disease.

To manage the effects of anthropogenic activities on wild species, it is important to be able to identify population structuring, and infer population connectivity. It is important to do this accurately, and with enough resolution to be able to detect changes in structuring over time and across spatial scales (Margules and Pressey, 2000, Naidoo *et al.* 2006, Rodrigues and Brooks, 2007).

The results discussed in Chapter 3 and 4 of this thesis indicate that relatedness is generally higher within bioregions (areas with similar habitat profiles), regardless of geographic distance (Kjeldsen *et al.* 2019). While across a national/species wide scale, broad-scale population differentiation is primarily driven by an isolation-by-distance genetic structure model (49-69% of genetic variance, (R^2=0.695), with clinal local adaptation corresponding to habitat bioregions (Kjeldsen *et al.* 2019). However, only weak levels of IBD were observed between regions, and no strong signatures of IBD were observed within populations (R^2= 0.001–0.158). Other environmental factors, or barriers and resistors to dispersal, were more informative than straight-line distances to describe observed patterns of genetic structuring within all regions. Population structuring is likely to be a result of complex interactions between several biotic and abiotic factors, however the most significant influencing factors appear to be directly linked to habitat type (Kjeldsen *et al.* 2019). Perhaps a limit to dispersal is not distance alone, or just the presence of eucalypt species, but rather a combination of the plasticity of the microbiome, coupled with suitable food trees. The results presented here support the idea that dietary preferences are not easily changed. One area of future study should examine if the settlement of young males in new regions is dependent on food tree tolerance, or if they are able to adapt to new areas if needed. If they are able to adapt, at what spatial scale might they be limited? A future area of study may examine if the diversity in eucalypt species (and palatable food trees) is linked to a koala's microbiome, and if this affects its ability to disperse to different areas. This will be particularly important in the coming years, as vegetation structure will likely change drastically as a result of both climate change and recent severe bushfires throughout Australia (Bennett *et al.* 2020, Jalaludin *et al.* 2020, Sullivan, 2020).

Taxonomic resolution

Identification of gene flow and physical barriers to dispersal are important to aid overall species management. Similarly, the identification of taxonomic or biological barriers is also important – particularly to developing appropriate management legislation (Dubois, 2003). The definition of species and sub-species is somewhat subjective, with many definitions having previously been suggested. In this thesis, I suggest a model/definition that is in keeping with the biological-phylogenetic species concept (Cracraft, 1987), where a sub-species is defined by: no significant admixture between populations, and evidence of the groups being on "separate evolutionary paths".

Dividing koalas into multiple sub-species may have been simpler from a legislative point of view in the past (i.e. state by state), however given there is no evidence of speciation in extant koala populations (Houlden *et al.* 1999, Johnson *et al.* 2018, Kjeldsen *et al.* 2019, Kjeldsen *et al.* 2016), koalas should be

considered as a single species group, and managed at a regional level (Kjeldsen *et al.* 2019). However, koala numbers are highly variable across the species range, and so management at a species level would not be ideal. For the purposes of IUCN/endangered listings, it is possible that koala populations should be classified into two groups – at risk, and least concern, with these listing considering factors such as overall numbers, population densities, and genetic diversity.

Incorrect assignment to a population, or sub-species group, can lead to mismanagement of conservation programs, with several downstream effects. Divergent populations may be better adapted to different climates or habitats, and so these groups should be identified to tailor management to the specific group. While no evidence of speciation was observed in this thesis, population structure was linked to bioregion, which may serve as a proxy for "sub-species" for management purposes. The overall aim of any management plan should be to ensure that diversity is maintained, and to preserve unique adaptations to as many environments as possible (Norris, 2004, Joseph *et al.* 2009).

Accurate species classification also reduces the incidence of both over, and under-grouping populations. Splitting groups can be problematic, particularly in non-model organisms, as this not only fragments populations, but also spreads resources too thinly (Naidoo *et al.* 2006). Conversely, over-grouping populations can lead to the loss of unique sub-species. Over grouping may be a good way to preserve resources in management plans, however this can lead to important groups/populations being overlooked and subsequently lost (Feckler *et al.* 2014). In the koala, signatures of selection are clearly evident between different bioregions/habitat types throughout Australia, and over grouping could lead to admixture between these regions – and a reduction in region-specific adaptations.

Ultimately, there is a need for balance when classifying groups of individuals for management or re-introduction purposes. While it is important to identify and protect threatened populations, limited funding for these projects means that it is often more practical to prioritize efforts where they will have the biggest overall impact, rather than expending resources on specific areas. In the case of the koala, perhaps grouping by bioregions, and then focusing efforts on areas of higher diversity, may allow for the most effective conservation at a species level.

Genetic Diversity

In addition to providing genomic resources, and reviewing the species classification of the koala, a major aim of this thesis was to assess overall genetic diversity of koala populations across the species range, and to determine areas of interest (either with low or high diversity levels). Previous work encountered difficulties in comparing populations, as the primary method of genetic analysis was to use mitochondrial DNA, or microsatellites, at a small geographic scale (Cheng *et al.* 2018, Cocciolone and Timms, 1992, Dennison *et al.* 2017, Houlden *et al.* 1999, Houlden *et al.* 1996b, Houlden *et al.* 1996d, Jobbins *et al.* 2012, Lau *et al.* 2014, Lee *et al.* 2010a, Lee *et al.* 2012a, Lee *et al.* 2010b, Lee *et al.* 2012b, Wedrowicz *et al.* 2018). This method is useful, however in a species with low mitochondrial

variation, like the koala, detecting differences between regions has proved difficult (Johnson *et al.* 2018, Wedrowicz *et al.* 2018). Additionally, studies prior to this thesis relied heavily on neutral genetic markers alone, which do not allow for detailed investigation of signatures of selection in the genome (Kjeldsen *et al.* 2016). Genome-wide SNPs have provided a novel opportunity to investigate not only neutral differences between regions, but also allowed preliminary study of genetic-environment interactions. Importantly, a major result of this thesis was the identification of a population of koalas with high genetic diversity, which appears to hold signatures from both northern and southern populations of koalas (Kjeldsen *et al.* 2019, Kjeldsen *et al.* 2016).

The populations surrounding the Sydney Basin region are particularly interesting as they hold much of the diversity of the species (Kjeldsen *et al.* 2019), and are within a phenotypic "transition zone" for the species. I theorize that this population may contain remnant diversity which has been lost from Southern populations due to hunting/population crashes. As discussed in Chapter 3, it also appears that this region may have contained a historic barrier to dispersal, in that phylogenetic analysis indicates a clear (if shallow) division in this region. However, admixture is currently high in this region, indicating that the historic barrier is no longer present. This is interesting because while the interbreeding of divergent populations can increase diversity overall, which is what is observed in this region, this diversity "hotspot" does not appear to have had time to disperse further than central NSW populations. With northern NSW and QLD populations being genetically distinct from the Sydney region. This region is highlighted as it should be studied more extensively, and carefully monitored as it may provide a diversity reservoir for the species as a whole.

Genomic resources

If a species is under threat from outside factors (e.g. urbanisation leading to habitat loss or fragmentation), limited genetic diversity can mean that populations are more likely to be negatively affected by stochastic events (Clavel *et al.* 2011). If we are to reduce the negative impacts associated with these threats, a comprehensive knowledge base, and access to resources is a primary requirement. In order to effectively manage a species we need resources which allow us to assess overall genetic diversity (potential to adapt). If a species has reduced or limited genetic diversity, it is unlikely to be able to adapt to changes in the environment or withstand stochastic events.

Providing an easily accessible, affordable, and standardized genomic resource for a non-model organism has been challenging in the past (Guppy *et al.* 2018). Funding for the conservation of wild (non-model) organisms is often difficult to obtain, and tends to be spread thinly across many species. For this reason, many researchers are reluctant to share their existing resources, meaning that new researchers may develop similar resources multiple times, in ways that mean the resulting research cannot be directly compared (i.e. Developing multiple panels of SNPs, or multiple sets of microsatellites). If a universal genomic resource is to be utilized in the future, it must have several key

traits; utility in estimating key diversity metrics, utility across the species distribution, affordability, and accessibility.

Firstly and most importantly, the resource needs to be useful and widely applicable. The resource must provide most (if not all) of the commonly estimated metrics needed to inform effective management. The resource should be able to estimate parameters such as relatedness (both at a parent-offspring, and population level), population of origin, and genetic diversity. These are key parameters which allow for prioritization of funding resources, and tracking levels of inbreeding in captive populations over time. The resource must also be applicable across the entire species range. There is high variability in diversity levels across koala populations (Kjeldsen *et al.* 2019), and different ecological histories make direct comparisons difficult – so a useful resource should take this into account.

The results presented in Chapter 2 and 5 provide the first examples of a genome-wide resource which is also relevant to the entire species distribution. In Chapter 5 specifically, I have identified 1,850 candidate SNP markers, which are informative to assess genomic diversity, can accurately assign parentage, and can assign individuals to wild source populations from across the species range. The pedigree errors identified in the captive population sampled highlights the need for alternative methods of tracking breeding programs. These markers have been tested in all major koala populations across Australia, and also in a captive population at San Diego Zoo. This demonstrates their usefulness in both in situ, and ex situ management programs. The resource provided here has identified suitable markers for answering a variety of ecological questions, facilitating ongoing management of wild populations. However, it should be noted that active management of koala populations requires ongoing sampling, which can be costly and logistically difficult at a large scale. Further development of these markers into a solid state genotyping array would increase the utility of this resource by allowing the genotyping of lower quality samples, such as scats (Schultz *et al.* 2018). Many GBS approaches still rely on high quality, undegraded DNA to successfully genotype individuals, but obtaining fresh tissue or blood samples from wild koalas is logistically difficult (Schultz *et al.* 2018, Schultz *et al.* 2019). A solid state array would facilitate the ongoing monitoring of koala populations by allowing more non-invasive sampling strategies to be used.

The resource should also be widely accessible. A hesitation to publicly share all aspects of research is understandable because these resources are extremely time consuming and complicated to develop. While reputable publishers require complete disclosure of data and methods, it is still possible to achieve this while still making the published data difficult to reproduce.While this is undoubtedly unethical, it does not appear to be uncommon. However, if the aim of the research is to benefit conservation, then collaborative approaches are far more successful and effective in the long term (Caudron *et al.* 2012). Access to resources should be freely available to all researchers, and conservation groups, to encourage an exchange of information at all management levels (research, zoos, wildlife

carers, governments). The results presented in this thesis offer the first steps to develop a universal genomic testing panel for koala management, which will be made freely available to other stakeholders for future use.

Overall Recommendations, Future direction, and Final comments

The primary aim of this thesis was to examine the genetic ecology of koalas across their current geographic range, and to develop and provide resources to inform the management of this species. Considering the iconic nature of the koala, and their broad geographic range, any attempt to achieve this was a significant task. Obtaining the number of genetic samples required for a robust dataset would have been challenging for any wild species, but was particularly difficult in a charismatic, cryptic folivore such as this. This was not only due to the difficulty of tracking and sampling wild koalas, but also because of the politically charged environment found in many wildlife research areas. This reluctance to share information severely hinders significant progress in management. Without widespread collaboration between researchers, and the involvement of stakeholders at all levels, projects such as these will invariably fail to produce meaningful results. A publicly accessible database, supported by government, conservation groups, and researchers, may offer a solution to this.

Collaboration and sharing of resources will be imperative to the successful management of populations affected by the 2019-2020 Australia bushfires. This disaster impacted many of the koala populations studied in this thesis, with the damage being severe in regions which were shown to contain high levels of diversity (i.e. The Sydney basin region, and Port Macquarie). The original aim of this thesis was to provide a tool for continuing management of koalas across Australia. While population structure and connectivity may change as habitats recover from the widespread damage, the resources provided here will allow for not only ongoing population monitoring, but a comparison to pre-fire population dynamics in this species.

The results discussed in this thesis provide a practical resource, and the foundations for robust and comparable genetic monitoring of koala populations across Australia. By developing resources which are reliable, and widely accessible, I hope that this will encourage future collaborations, and lead to effective conservation of koalas in the future.

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