Investigating Australian mammal extinctions and conservation using ancient DNA, population genetics and time-series analysis

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Thesis Abstract

Biodiversity loss is a global problem with potentially catastrophic consequences for ecosystem function, human health and economics. Australia has one of the highest rates of species extinctions in the world, and the continents' unique mammal fauna have suffered disproportionately. To minimize further mammal extinctions, conservation efforts should be focused in areas where they can be most effective. These efforts can be assisted by research that investigates species extinction risk in changing environments and that identify strategies to minimise biodiversity loss with limited resources. Extinction science is a multi-disciplinary field that aims to improve our understanding of extinction risk and the conservation interventions that can alleviate it. The field incorporates ideas and data from palaeontology, field studies and genetics to understand all aspects of species declines and to apply this understanding to conservation efforts.

In this thesis I aim to improve our understanding of Australian mammal extinction and declines, and to apply current knowledge to aid future conservation efforts. I answer a variety of questions related to Australian mammal extinction and conservation by analyzing ancient DNA, population genetic and radiocarbon age time-series datasets.

Specifically, I use:

- Genetic population assignment to test the origins of a putative relict population of the Tasmanian devil (*Sarcophilus harrisii*) on mainland Australia—Chapter 2.
- Time-series analysis to validate the common assumption of synchronous extinction of the Australian mainland devil and thylacine (*Thylacinus cynocephalus*)—Chapter 3.

- Ancient DNA analysis to reconstruct the phylogeography and demographic history of thylacines leading up to their extinction on both the Australian mainland and Tasmania—Chapter 4.
- High-resolution genetic monitoring to evaluate the success of reintroduction programs in maintaining the genetic diversity of four species of threatened Australian mammals: The greater stick-nest rat (*Leporillus conditor*), the western barred bandicoot (*Perameles bougainvile*), the burrowing bettong (*Bettongia lesueur*) and the geater bilby (*Macrotis lagotis*)—Chapters 5 and 6.

Ultimately, I resolve several natural history questions that have conservation implications for Australian mammals today, using a variety of cutting edge technologies and analytical methods.

Thesis Declaration

I, Lauren C. White, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Lauren C. White Date

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Chapter 1

General Introduction

Chapter 1. General Introduction

Extinction Science and Conservation

The modern rate of species extinction vastly exceeds the long-term background rate and many scientists argue that we are entering a sixth "mass extinction" caused by human activity (Ceballos *et al.*, 2015). This decline in biodiversity is largely recognized as undesirable, not only from an ethical stand-point, but also for its negative impact on ecosystem functioning and human well-being (Cardinale *et al*., 2012; D, 2 *et al.*, 2006). In 2002 world leaders committed, through the United Nations Convention on Biological Diversity, "to achieve by 2010 a significant reduction in the current rate of biodiversity loss" (Balmford *et al.*, 2005; European Council, 2001). Despite several achievements, that target was unequivocally not met and the revised 2020 targets also seem unlikely to be realised (Butchart *et al*., 2010; Tittensor *et al.*, 2014).

To reverse this trend large policy changes are needed, which can be assisted by research into how best to focus conservation efforts. For example, understanding extinction risk under future environmental change is essential for focusing funding and maximising the conservation of biodiversity.

Extinction science is a still forming field of research that aims to improve our understanding of extinction risk and conservation interventions that can alleviate it. It incorporates ideas and data from palaeontology, ecology, field studies and genetics to understand the patterns and processes of species extinction and survival (i.e. why some species survive while others do not). The field has two main objectives: a) to investigate and understand species extinctions and declines, and b) to use this information to improve conservation efforts to minimise human-induced extinctions.

Here, I summarize current methods of estimating extinction risk and, how studying prehistoric and modern species extinctions and declines improves extinction risk predictions for conservation. I then examine extinction science in an Australian context and discuss how this field of research can contribute to the preservation of Australia's unique fauna.

Current Methods of Estimating Extinction Risk

It is well established that there is variability in species extinction risk and that several individual and population traits are good indicators of vulnerability. Large body size, high trophic level, specialized habitat needs, poor dispersal ability and—at a population level—low effective population size and spatial rarity, are often-cited traits that confer greater extinction risk (McKinney, 1997; Kotiaho *et al*., 2005). These traits have been found generally to increase extinction risk in both the paleontological record and in modern extinctions, which so far "conform mainly to intensified versions of background expectations" (Jablonski and Chaloner, 1994). However, the effect of such traits varies between taxonomic groups, habitat types, spatial scales and the external mechanism (for example the invasion of a competing species, or climate disruptions) of population decline (Bennett and Owens, 1997; Cardillo *et al*., 2005; Fritz *et al*., 2009). Additionally, many of these traits are not independent and interact in complex ways with each other and other external and stochastic factors (Davies *et al.*, 2004; Olden *et al.*, 2008).

Several methods have been developed to model these complex interactions and predict extinction risk, such as population viability analysis (PVA) and environmental niche modelling (ENM). PVA brings together measures of environmental variability and species life history characteristics to simulate population change and forecast extinction risk within a

given number of years (Brook *et al*., 2000). ENM uses information about a species dispersal ability and current distribution in environmental space to predict whether suitable habitat will be available to them under different climate and environmental change scenarios (Martínez-Meyer *et al.*, 2004).

While both methods have been invaluable in guiding decision making for conservation, they can be subject to much uncertainty. The range of estimates for total extinctions due to contemporary climate change remain troublingly large (Urban, 2015) and the uncertainty in PVA predictions when less than 10 years of demographic data are available can restrict their usefulness (McCarthy *et al*., 2003). Such uncertainty can be due to the explicit modelling of stochastic variables, but uncertainty in input data and un-modelled processes can also produce noise and error. To improve our ability to estimate extinction risk, further studies are needed on the mechanisms and patterns of past species extinctions and declines.

The Paleontological Record and Prehistoric Extinctions

Investigating past extinctions can help us understand contemporary species extinctions and declines in several ways. Firstly, using the paleontological record can be a more time-efficient alternative to long-term monitoring of modern taxa. Many species are in urgent need of conservation action, but limited monitoring data can hinder PVA and create uncertainty about what action to take (McCarthy *et al.*, 2003). Using the fossil record of the same or related species during past times of rapid change can provide hundreds to thousands of years of information for use in predictive models (Davies and Bunting, 2010).

Secondly, the paleontological record can be used to test extinction risk models currently in use. The few cases to do so already show that the predictive capacity of ENMs break down

under large changes in global mean temperature (Pearman *et al.*, 2008; Varela *et al.*, 2011; Veloz *et al.*, 2012), likely because the models were extrapolated beyond the data used to build them (Fordham *et al.*, 2016). For example, models are particularly prone to erroneous results in cases where the there is no modern analogue to the past climate conditions (Roberts & Hamann, 2011). Roberts & Hamann (2011) showed that such 'no-analogue' climate occurs over most of North America past 21,000 BP and also in the Rocky Mountains during the mid-Holocene warm period (6,000 BP). Fordham *et al.* (2016) suggest that a method in which models of varying complexity are built and tested against prehistoric, climate-driven range collapses and population declines could provide deeper understanding of the causes of errors in projections.

Finally, contrasting species that went extinct with those that didn't during times of rapid change can provide a mechanistic understanding of how ecological traits interact and relate to extinction vulnerability (Kiessling and Kocsis, 2016). Fossil records since the Late Pleistocene are particularly useful for understanding future species response as this period includes the peak of the last glacial maximum (LGM, ~20,000), the subsequent rapid warming and the proliferation of human populations around the globe (Koch and Barnosky, 2006).

These applications of the fossil record to extinction science require accurate and precise estimates of extinction time across fossil taxa. Additionally, they often require an understanding of the possible mechanisms that may have drove prehistoric extinctions, the palaeoecological environment in which they occurred, and the demographic patterns of declines.

Estimating Extinction Time

Determining when a species became extinct is critical for testing alternative hypotheses of extinction drivers, but is not a trivial matter and becomes increasingly difficult with the passing of time (Fagan and Holmes, 2006). First, reliable fossil ages must be obtained. All methods for dating material of interest are based on measuring naturally occurring elements that decay at predictable rates. For example, the most widely known technique is radiocarbon dating, which can be used on materials up to ~55,000 years old (Ramsey *et al.*, 2007). The reliability of the estimate of a fossil's age is dependent on many factors including the dating technique, pre-treatment protocols, material quality, whether the dating was direct (dating of the fossil material) or indirect (dating of other material that is associated in time with the fossil), and—in the case of indirect dating—how strong the association is (Saltrr *et al.*, 2015). Uncritical use of fossil ages can result in inaccurate estimates of events and therefore, several quality rating systems for published dates have been developed (Pettitt *et al.*, 2003; Rodríguez-Rey *et al.*, 2015).

Robust estimates of a species' extinction time require many dated fossils sampled over an appropriate period. The age of the youngest fossil and the true extinction time of the species will inevitably diverge due to incomplete sampling, taphonomic bias (biases arising from variation in the process of fosillisation) and uncertainty in radiometric dating (Signor and Lipps, 1982; Solow et al., 2006). To address these problems, many statistical models have been developed to estimate extinction time (and the associated uncertainty) using a time series of fossil ages (Saltré *et al.*, 2015). For example, such methods were used by Perry *et al.*, (2014) to show that the New Zealand moa (Aves, Dinornithiformes) became extinct synchronously across sites surveyed, despite being separated by hundreds of kilometres, and large differences in size between species. The accuracy of these methods depends ona number of characteristics of the time series being evaluated and different models may be

preferred based on these chraracteristics (Saltré *et al*., 2015) Saltré and collgues (2015) developed a decision tree to help researchers decide which model is most appropriate for their data.

Paleo-Environmental Reconstruction

After a reliable estimate of extinction age has been acquired, extinction time is usually considered in the context of paleo-ecological or paleo-climatic events, to determine the cause of decline. Paleo-climate is generally reconstructed using proxies whose physical characteristics, such as isotopic composition, are influenced by the climatic conditions of the time in which they were deposited or grew. Examples include ice cores, tree rings, corals, lake and ocean sediments and carbonate speleothems (IPCC, 2001). Paleoecological proxies, such as sub-fossil pollen, diatoms, isotopes and plant macro-fossils, can be used in similar way to establish biotic compositions and transitions during pre-history (Seddon *et al.*, 2014). For example, Miller *et al*., (2016) used stable isotopes measured from fossil emu (*Dromaius*) egg shells to document a loss of C4 grasses in the Australian arid zone 50,000 years ago, coincident with the extinction of the giant megafaunal bird, *Genyornis*.

Demographic History and Ancient DNA

Estimating demographic history from the fossil record alone relies on consistent rates of bone preservation through time, a prerequisite that is often not met due to taphonomic bias. Genetic studies can bridge this gap by relating patterns of diversity written in the DNA of a sample to the demographic history of the population from which it was taken. This is usually done using coalescent theory: a stochastic process that describes how population processes shape the genealogy of sampled DNA sequences (Kingman, 1982).

Ancient DNA (aDNA)—DNA extracted from preserved remains of organisms—is a powerful tool in this framework. It can be used to reconstruct the demographic history of extinct species and extant species for which the genetic patterns have been eroded in modern populations by bottlenecks or introgression (Chang and Shapiro, 2016). For example, Stiller *et al.*, (2010) compared the population size trajectories of the extinct cave bear and extant brown bear in Europe using aDNA in a Bayesian coalescent framework, and found that while the population size of brown bears has been constant over the last 60,000 years, the decline of cave bears began 25,000 years before their extinction. The beginning of this decline does not correlate with any major climate event, and the authors suggest that competition for cave habitat with humans and Neanderthals may have been a factor in their decline.

Working with aDNA can be challenging: degrading processes after the death of an organism lead to DNA fragmenting into increasingly small pieces, cross-links between DNA strands that prevent amplification, and the hydrolytic deamination of bases which are then misread during sequencing (Dabney *et al*., 2013). Additionally, ancient samples usually contain very low quantities of endogenous DNA. To avoid contamination from modern DNA and PCR products, aDNA samples must be processed in specially designed clean facilities with stringent decontamination protocols (Cooper and Poinar, 2000).

These difficulties restricted early aDNA analyses to short sections, often less than 100 bp in length (Hagelberg *et al.*, 2015). Over the last decade, however, advances in sequencing technology have revolutionised the field. Using next generation sequencing (NGS) technologies, which are often designed specifically for short reads, it is now possible to sequence a large number of DNA molecules quickly, accurately, and at a comparatively low cost per base (van Dijk *et al*., 2014).

Sequencing a random sample of molecules from a DNA extract (termed shotgun sequencing), is the most straight-forward approach to NGS. However, the low endogenous

content of aDNA can often make shotgun sequencing uneconomical (Knapp and Hofreiter, 2010). Hybridisation enrichment allows the relative increase in concentration of target DNA molecules in an extract before sequencing (Gnirke *et al*., 2009; Hodges *et al*., 2009). Synthetic RNA baits that are complementary to the target regions are incubated with molecules from the DNA extract, resulting in target molecules annealing to the baits. Off-target molecules can then be removed and the target-enriched DNA released from the baits. Hybridisation enrichment and NGS thus open up a range of possibilities for genomic-scale studies of aDNA, including whole mtDNA genomes (Llamas *et al*., 2016; Mitchell *et al*., 2016; Soubrier *et al.*, 2016) and hundreds of nuclear genes (Bailey *et al*., 2016; Bi *et al*., 2013; McCormack *et al*., 2016).

Contemporary Species Declines

The accelerated rate of human-induced species loss makes it possible to study species declines as they happen. They can be used in the same way as the fossil record to inform, test and improve models, particularly when long-term monitoring data are available, but also highlight several central factors that often play a role in species decline, that are difficult to study using the fossil record alone.

Disease

Evidence for parasite or pathogen-driven species declines are nearly impossible to detect in the fossil record as traces of the pathology are usually lost to time (Kathleen Lyons *et al*., 2004). We know however, from recent species declines, that disease can pose a significant threat to natural populations (De Castro and Bolker, 2005). Pedersen *et al*., (2007) found that wild species that are related to widespread domesticates, eg. dogs, cats, goats, sheep, cattle

and pigs, were more likely to be threatened by infectious disease, likely due to the increased chance of cross-species infection. This type of information can be incorporated into extinction risk assessments to improve their accuracy.

Behaviour

Fossils can tell us nothing about mate choice, parental care, intraspecific behaviour, reproductive skew, communication, kin recognition or personality differences (Caro and Sherman, 2011). It is only through studying extant species that we can understand how these types of traits effect extinction risk. For example, species with complex social structures for mating, group foraging or group defence have been shown to be more vulnerable to extinction because their persistence depends upon a larger unit than the individual (Courchamp *et al*., 1999).

Inbreeding Depression

Inbreeding has been known to cause deleterious effects since Darwin's time. Darwin showed strong negative impacts of inbreeding in over 50 species of plants, and he often worried about the consequences of marrying his first cousin (Darwin, 1876; Moore, 2005). Inbreeding redistributes the frequency of genotypes in a population by increasing the proportion of homozygotes (and correspondingly decreasing the proportion of heterozygotes). This can cause the increased expression of deleterious traits (i.e. inbreedind depression), which are mostly recessive. Alternatively, if a heterozygote is more advantageous than either homozygote genotype, inbreeding depression will be caused by reduced opportunities to express this overdominance. Some evidence exists for both mechanisms, although it can be difficult to distinguish the two (Keller and Waller, 2002).

Furthermore, inbreeding depression is likely a multi-locus characteristic in most species, meaning both mechanisms could be at play simultaneously (Charlesworth and Willis, 2009).

Inbreeding depression has been linked to population declines and/or extinctions in both the wild (Crnokrak and Roff, 1999; Madsen *et al.*, 2004; Saccheri *et al.*, 1998) and captive (Kalinowski *et al*., 2000; Laikre and Ryman, 1991; Ralls and Ballou, 1983) populations. Additionally, Brook *et al*. (2002) and O'Grady *et al*. (2006) confirmed, using computer simulations, that inbreeding depression has a significant impact on extinction risk and advocated for its inclusion in all PVA analyses. Inbreeding depression is of particular concern for conservation as mating with relatives is unavoidable in small unmanaged populations (Frankham *et al*., 2010). This realization prompted many conservation programs to adopt the preservation of genetic diversity as an explicit goal (Laikre, 2010; Laikre *et al*., 2010).To meet this target, it is important for inbreeding and genetic diversity to be monitored in managed populations.

Inbreeding is measured by the probability that two alleles in an individual are identical by descent, and is denoted as F. This can be calculated from pedigrees where available. For example, an offspring of a full-sibling mating will have a 25% chance of receiving the same allele from each parent at any locus, and so their F=0.25. However, pedigrees are only available in very select cases in which a population has been intensely managed or monitored.

Alternatively, inbreeding can be estimated from genetic markers by assuming that heterozygosity is negatively correlated with inbreeding (Lynch and Ritland, 1999). Genetic marker measurements of inbreeding are arguably faster and less expensive than pedigree data because they do not require records collected over multiple generations. However, microsatellite based measurements (the staple of population genetics for the last two decades) have a low correlation with pedigree based values and are less able to detect inbreeding

depression (Pemberton, 2004). This is not surprising because F can have high locus to locus, and inter-individual variation due to recombination and Mendelian segregation (Hill and Weir, 2011). While pedigree measures of F represent the expected average, microsatellites measure only a limited number of loci across the genome.

Large single nucleotide polymorphism (SNP) datasets are more genome-representative than microsatellites and, thanks to NGS, are being increasingly used for conservation genetic studies. Using methods such as restriction site-associated sequencing (RAD-seq) thousands of SNPs can be sequenced in non-model species without the need for a lengthy marker development stage. A number of studies have shown that inbreeding estimated using large SNP datasets is just as, and in some cases more, accurate as using pedigree data (Hoffman *et al.*, 2014; Kardos *et al.*, 2015; Wang, 2016).

Extinction and Conservation in Australia

Australia has a unique fauna with a high level of endemism. Approximately 84% of Australian mammals, 45% of birds and 89% of reptiles occur nowhere else in the world (SoE Committee, 2011). This can be attributed to the continent's long geographic isolation, tectonic stability and unique climatic history. Unfortunately, Australia also has one of the highest rates of recent species loss. While this most recent wave of species declines in Australia is arguably the continent's most severe, it is not the first.

Late Pleistocene Megafaunal Extinctions

During the late Pleistocene (between 130,000 and 10,000) almost all of Australia's megafauna (animals > 44 kg) became extinct. This includes the marsupial giants, such as

Diprotodon optatum, and *Thylacoleo carnifex*, as well as the giant snake *Wonambi naracoortensis*, the giant crocodile *Pallimnarchus pollens*, and the giant flightless bird *Genyornis newtoni* (Wroe *et al.*, 2013). The cause of these extinctions has long been debated and remains controversial. The first hypothesis proposes that humans (who arrived in Australia ~50,000 years ago) caused the megafaunal extinctions through over hunting or changed fire regimes (Flannery, 1994; Miller *et al.*, 2005). The alternate hypothesis is that climate change caused staggered extinctions over several glacial cycles (Webb, 2008). To test these hypotheses, extinction times across taxa have been tested for correlation with the timing of human arrival, and paleoecological and climate records. Research and debate continue around the validity of different fossil ages, extinction time estimates, proxies, and archaeological and genetic evidence (Johnson *et al.*, 2016; Saltr6 *et al.*, 2016).

Late Holocene Extinctions

A second, smaller, but no less enigmatic, wave of extinctions occurred during the Late Holocene on mainland Australia. The thylacine (*Thylacinus cynocephalus*) and devil (*Sarcophilus harrisii*) were Australia's largest remaining carnivorous marsupials. Although inferences are difficult due to the paucity of the fossil record, they appear to have been widespread across most of the continent during the Late Pleistocene (Brown, 2006; Owen, 2003). They disappeared from mainland Australia in the Late Holocene, along with a small flightless bird, the Tasmanian swamphen (*Gallinula mortierii*), surviving only on the island of Tasmania (Baird, 1991; Johnson, 2006).

Based on the timing of events and Tasmania's isolation from the likely impacts, three competing (but not mutually exclusive) hypotheses for the cause of these extinctions have been proposed. The first puts the blame on the dingo (*Canis lupus dingo*), a novel competitor and/or predator of the thylacine, devil and swamphen (Corbett, 1995). Dingos are

descendants of early domestic dogs in Asia and were introduced to Australia 4,000-5,000 years ago. They never reached Tasmania, which was already isolated from the mainland at that time. Johnson and Wroe (2003) proposed an alternate hypothesis: that the extinctions were due to an increase in human impact. Beginning around 5,000 years ago archaeological evidence on mainland Australia points to an increase in the human population size, a less nomadic lifestyle in many regions, and changes in hunting practices, including an increased use of stone tools. These trends were markedly absent in Tasmania, where human population size remained low. Alternatively, Brown (2006) suggested a role for climate in these extinctions. Following the relatively wet and stable period of the Holocene optimum (~8,000–5,000 years BP), a strengthening of the El Niño Southern Oscillation (ENSO) caused a shift in Australia's climate towards a drier, more drought prone system. The effects of increased ENSO activity are assumed to have had a minimal impact on Tasmania due to its maritime climate and consistent rainfall (Donders *et al*., 2008). Disentangling the effects of climate change, invasive species and altered land use in species extinction risk are important for predicting future species extinction risk in Australia.

Species Extinctions and Declines since European Arrival

Australia has one of the highest rates of species extinctions and declines in the world. Approximately 41 of Australia's faunal species have become extinct in the last 200 years and 13% of Australian terrestrial vertebrates are threatened with extinction. This does not include the numerous Australian species which have experienced local extinctions on the mainland, surviving only on offshore islands. As seen in most regions, Australia's mammals have suffered particularly badly. Twenty-eight terrestrial mammal species have become extinct since 1788 (the last in 1991), a further nine are extinct on the mainland and survive only on offshore islands, and another ten are critically endangered (Johnson, 2006).

These Australian mammal extinctions have been attributed to disease, competition with introduced herbivores (especially sheep and rabbits), loss of habitat due to changed land use, introduced predators and direct persecution by Europeans. This long list of pressures occurred over much of the continent near-simultaneously after European arrival and disentangling the relative effects on any one extinction or decline is difficult (Johnson, 2006). However, a growing consensus in the scientific literature is that two introduced predators, the feral cat and red fox, had and are still having a decisive influence on the extinction and decline of most Australian fauna, especially terrestrial mammals in the critical weight range of 35-5500 g (Johnson, 2006; Woinarski *et al.*, 2015). Based on these findings, many strategies have been employed to reduce the impact of feral predators, including shooting, trapping and poison baiting. The best protection is afforded by offshore islands and fenced reserves from which predators can be exterminated and excluded (Dickman, 2012).

Climate change will create added threats to Australian species and exacerbate those already in place. Climate projections for Australia confirm that most of the changes observed over recent decades, such as increased average temperatures, lower average rainfall and more extreme weather events, will continue and intensify in the future (CSIRO and Bureau of Meteorology, 2015). Modelling predicts severe to catastrophic losses of Australian species that inhabit tropical savannahs, coral reef systems, alpine environments, high-altitude tropical forests and coastal or island environments (Laurance *et al.*, 2011).

Conserving Australia's biota is already a challenge and will become even more so as the impacts of climate change become more severe. Current conservation action in Australia is extremely varied and encompases a multitude of people, approaches, policy, organiziations, stake-holders, and areas (protected or otherwise). Continued research into how conservation actions and interventions can best alleviate a species risk of extinction is necessary to minimize future species loss in Australia.

Summary and Thesis Outline

The current rate of species extinctions is far above the average background rate. To focus conservation efforts to where they are most needed, a better understanding of species extinction risk and conservation interventions to alleviate them, is needed. To this end, the field of extinction science aims to better understand past and contemporary species decline. Past extinctions can provide critical information about characteristics that infer greater extinction risk, and can be studied using radiocarbon dating, paleoecological and paleoclimatic proxies, and ancient DNA. However, some characteristics can only be examined in extant populations, such as disease impact, behavior and inbreeding depression.

Australia has one of the world's worst histories of species extinctions, and the continents unique mammal fauna have suffered particularly bady. Conservation action continues to be critical to many species survival and research into extinction risk can best be alleviated is necessary.

In this thesis I aim to use several methods to improve our understanding of past extinctions, monitor changes in threatened species today and ultimately aid future conservation efforts. I focus on Australian terrestrial mammals, a group with particularly bad records of extinctions and declines. Below I outline the aims of my five data chapters, which address various questions about extinction and conservation of Australian mammals:

Data Chapter Summaries

Chapter 2. Relict or reintroduction? Genetic population assignment of three Tasmanian devils (*Sarcophilus harrisii*) recovered on mainland Australia.

In this chapter I examine the possibility that a relict Tasmanian devil population could have survived on mainland Australia in modern times. Three devil specimens were collected from central Victoria between 1912 and 1991, two of which were found alive, raising the intriguing possibility that devils were not extinct on the mainland at the time of European arrival. This has important implications for proposed re-wilding conservation projects, which seek to reintroduce devils to mainland Australia as a means to control feral animal. Alternatively, these devils may represent recent, deliberate or accidental, translocations from Tasmania. I use an alignment of modern and ancient devil mitochondrial genomes to identify diagnostic SNPs that can distinguish between Tasmanian and ancient mainland populations.

Chapter 3: High-quality fossil dates support a synchronous, late-Holocene extinction of devils and thylacines in mainland Australia

In this chapter I examine and test the common, yet previously unvalidated assumption that the mainland extinctions of the thylacine and devil occurred at the same time, approximately 3,000 years ago. The alleged co-incidental timing of the mainland extinction events is taken frequently as evidence that they arose from a common cause that affected the highest trophic levels of the ecosystem. There is much interest in these extinctions because the hypothesized causes have parralells with processes threatening Australian mammals today (eg. introduced animals, change in human lifestyle and land-use, and climate change). However, the assumption of synchronous extinction has not been tested rigorously. I present high-quality radiocarbon ages of newly dated mainland devil and thylacine fossils and combine these with

reliable radiocarbon dates by quality-filtering published records for the first time. An ensemble-hindcasting approach is applied to these datasets based on five inferential methods to estimate extinction time for both species.

Chapter 4: Mitochondrial genome analysis reveals the demographic history and phylogeography of the enigmatic thylacines (*Thylacinus cynocephalus*)

The Tasmanian tiger, or thylacine, is an infamous example of a recent human-mediated extinction. Confined to the island of Tasmania in historical times, thylacines were rapidly hunted to extinction less than 150 years after European arrival. Thylacines were also once widespread across the Australian mainland, but became extinct there ~3,200 years before present (BP). Very little is known about thylacine biology and population history; the cause of the thylacines extirpation from the mainland is still debated and the reasons for its survival in Tasmania are unclear. Understanding why some populations go extinct when others do not is core to the field of extinction science. In Chapter 4 I investigate the phylogeography and demographic history of thylacines in Tasmania and the Australian mainland leading up to their extinctions in both locations using 51 new mitochondrial DNA (mtDNA) genome sequences obtained from sub-fossil remains and historical museum specimens.

Chapter 5: Evaluating the genetic consequences of reintroduction in four threatened mammal species and Arid Recovery Reserve

Genetic diversity is a vital aspect of reintroduction programs as low genetic variation can lead to reduced adaptive capacity, decreased population fitness, and increased risk of extinction. These problems are often exacerbated in reintroduced populations due to founder events, bottleneck effects, small population size and the isolated nature of sanctuaries. The Arid Recovery Reserve is an exclosure site in northern South Australia to which four native

mammal species—the greater stick-nest rat (*Leporillus conditor*), greater bilby (*Macrotis lagotis*), burrowing bettong (or boodie, *Bettongia lesueur*), and western barred bandicoot (*Perameles bougainville*)—were reintroduced 18 years ago. Tissue samples were taken from founder individuals so the reintroduced species provide a unique opportunity to study changes in genetic diversity through time in managed populations. In Chapter 5 I generate a large SNP dataset from samples from the current populations of all four species and from the founding individuals where available. I use this dataset to estimate the amount of genetic diversity lost and investigate selection in the reintroduced vs. source populations. We use this information to determine whether additional reintroductions are necessary at Arid Recovery and make recommendations for future reintroduction programs.

Chapter 6. High-resolution genetic monitoring and implications for conservation management of the greater stick-nest rat (*Leporillus conditor*)

The greater stick-nest rat (GSNR, *Leporillus conditor*) was formerly distributed through much of southern Australia, but was extirpated from the mainland in the 1930's due to changing land use and introduced animals. The species survived in a single population of ~1,000 individuals on the Franklin Islands off the west coast of South Australia. To alleviate the risk of total extinction, a captive breeding and reintroduction program was initiated for the GSNR in 1985, which has resulted in the establishment of five new populations. Despite this success, the recent demographic history of GSNRs poses several genetic threats to the future of the GSNR conservation program. In this chapter, we sequence thousands of SNP markers from individuals representing all extant populations of GSNR, and provide high-resolution information on genome-wide genetic diversity. The results are used to make recommendations and guide future conservation actions to maximise the preservation of genetic diversity and alleviate the risk of extinction in this species.

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Chapter 2

Relict or reintroduction? Genetic population assignment of three Tasmanian devils (*Sarcophilus harrisii*) recovered on mainland Australia

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Principal Author

Name of Principal Author (Candidate)	Lauren C White
Contribution to the Paper	Collected and analysed the data, wrote the manuscript and produced the figures.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/3/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Jeremy J Austin
Contribution to the Paper	Conceived the study, sampled museum specimens, interpreted the data and revised the manuscript.
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Relict or reintroduction? Genetic population assignment of three Tasmanian devils (*Sarcophilus harrisii*) recovered on mainland Australia

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Today, the Tasmanian devil (*Sarcophilus harrisii*) is found only on the island of Tasmania, despite once being widespread across mainland Australia. While the devil is thought to have become extinct on the mainland approximately 3000 years ago, three specimens were collected in Victoria (south-eastern Australia) between 1912 and 1991, raising the possibility that a relict mainland population survived in the area. Alternatively, these devils may have escaped captivity or were deliberately released after being transported from Tasmania, a practice that has been strictly controlled since the onset of devil facial tumour disease in the early 1990s. Such quarantine regimes are important to protect disease-free, 'insurance populations' in zoos on the mainland. To test whether the three Victorian devils were members of a relict mainland population or had been recently transported from Tasmania we identified seven single nucleotide polymorphisms (SNPs) in the mitochondrial genome that can distinguish between Tasmanian and ancient mainland populations. The three Victorian devil specimens have the same seven SNPs diagnostic of modern Tasmanian devils, confirming that they were most likely transported from Tasmania and do not represent a remnant population of mainland devils.

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1. Introduction

Lazarus taxa—species thought to be extinct but then rediscovered alive—stand in stark contrast to the current worldwide extinction crisis. The Australian vertebrate fauna has suffered a high rate of anthropogenic extinctions in the last 200 years, but also has a relatively high rate of species rediscovery [1]. Australian Lazarus species include the night parrot (*Pezoporus occidentalis*, rediscovered in 1990 after no sightings for almost 80 years) [2], the Dibbler (*Parantechinus apicalis*, presumed extinct in the wild for 63 years until an individual was captured in 1967) [3], Gilbert's potoroo (*Potorous gilbertii* rediscovered alive in 1994 after having been thought extinct for over 100 years) [4] and the tiger quoll (*Dasyurus maculatus*, observed on a motion-detecting camera in the Victorian Grampians in 2013, 141 years after it was last seen in that area) [5]. The rediscovery of a number of small to medium-sized mammals and birds in recent decades raises the possibility that other species have survived undetected.

The Tasmanian devil is a possible example of a Lazarus species previously thought to be extinct for thousands of years in part of its former range. Devils are the largest extant carnivorous marsupials and are currently found only on the island of Tasmania. Along with the thylacine (*Thylacinus cynocephalus*), devils were once widespread across the Australian mainland, but both species are thought to have become extinct there during the mid-Holocene (approx. 3000 years before present; [6,7]). More recent dates (430–630 years before present) for devil remains on the mainland have been rejected on methodological grounds [6].

Although early Europeans did not record living devils on the mainland, three devil specimens were collected in Victoria, in the southeast of the Australian mainland, between 1912 and 1991, two of which were found alive [8–10]. These discoveries raise the intriguing possibility that devils were not extinct on the mainland at the time of European arrival and that a remnant population persisted in Victoria until modern times. Recent examples of Lazarus species in Australia lend credibility to the idea that a relict devil population could have survived in the south-eastern corner of the mainland, a bioregion that resembles that of Tasmania [11]. Alternatively, the recovered Victorian devils may represent animals transported from Tasmania that were kept as pets or in wildlife parks, that subsequently escaped or were deliberately released. Museum specimens of all three animals are held at Museum Victoria, offering the opportunity to use preserved DNA to identify their origin.

Identifying the origins of the Victorian devil specimens has broader relevance than a simple natural history investigation. Understanding trends in wildlife trade and translocation are important for the design of quarantine regimes, which have become very important for Tasmanian devils since the onset of devil facial tumour disease (DFTD), a highly contagious cancer that has wiped out over 80% of the population [12]. The transport of devils out of Tasmania is now strictly controlled to ensure that 'insurance populations' of disease-free devils on the mainland are not threatened by DFTD. There are also scientific and public calls for devils to be translocated to the mainland as part of a rewilding programme to restore ecological functions to ecosystems dominated by introduced predators [11]. To date, none of these proposals have considered the impacts of translocating Tasmanian devils into areas potentially already occupied by relict populations of mainland devils. Therefore, for both insurance population management and rewilding proposals, it is critical to know if a remnant population of devils survives on the mainland, and whether this population represents an ancient relict or a recent introduction. To test whether the Victorian devils were members of a relict mainland population or had been recently transported from Tasmania, we analysed and compared mitochondrial DNA from the three museum specimens with data from Tasmanian (extant and subfossil) and mainland (subfossil) devils.

2. Material and methods

2.1. Samples and DNA extraction

Three devil specimens were collected in central Victoria between 1912 and 1991 and subsequently donated to Museum Victoria (figure 1, table 1). C6257 was caught and killed by a farmer in 1912 [9], C31255 was collected in 1991 and has skeletal damage that is consistent with road kill, and C22543 was caught alive in a rabbit trap in 1971 [10]. Samples of toepad (C22543), dried tissue (C31255) or tooth (C6257) were taken from the specimens and transferred to the Australian Centre for Ancient DNA (ACAD), at the University of Adelaide. We controlled for contamination of the museum samples with contemporary DNA and previously amplified mitochondrial DNA PCR products by conducting all pre-PCR work in a dedicated, physically separate, cleanroom facility, with the use of dead-air glove boxes

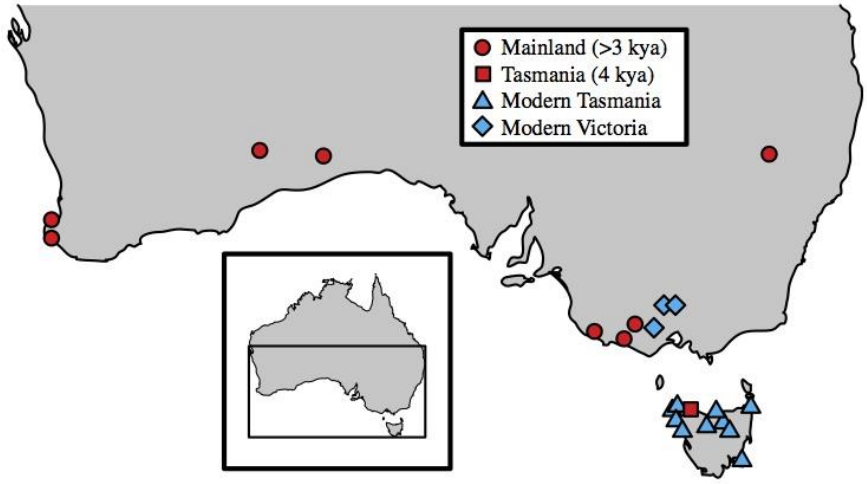

Figure 1. Map of southern Australia showing the collection locality for ancient mainland, modern Tasmanian and ancient Tasmanian samples used to identify geographically informative single nucleotide polymorphisms (SNPs), and the location of the three modern Victorian devil specimens of unknown origin.

Table 1. Museum specimen details for three Tasmanian devils collected in Victoria.

Museum Victoria accession number	date collected	specimen type	sex	locality	lat.	long.
C6257	1912	skin and skeleton	female	Tooborac	−37.05	144.78
C31255	28 March 1991	skin and skeleton	female	Faraday	−37.05	144.30
C22543	22 May 1971	skin	male	Dereel	−37.82	143.75

fitted with internal ultraviolet lights; regular decontamination of all work areas and equipment with sodium hypochlorite; personal protective equipment, and strict one-way movement of personnel. No contemporary devil samples or DNA had ever been present in the pre-PCR laboratory and we included a negative control alongside all extractions to monitor for contamination. DNA was extracted using the DNeasy Tissue Kit (Qiagen) according to the methods described by Boessenkool *et al.* [13] (tooth) and Austin *et al.* [14] (tissue and toepad).

2.2. Primer design and amplification

An alignment of 35 devil mitochondrial genomes including 18 Tasmanian, and 17 sub-fossil mainland samples (13 Tasmanian samples from [15], five Tasmanian and 17 mainland samples from A. Brüniche-Olsen *et al.*, unpublished data) was used to identify seven informative single nucleotide polymorphisms (SNPs) (figure 2) that distinguish between mainland devils, contemporary Tasmanian devils, and an extinct haplotype identified from a single ancient (approx. 4000-year-old) Tasmanian devil collected from Smithton. The mtDNA genomes from Brüniche-Olsen *et al.* (unpublished data) were generated from genomic DNA libraries enriched for mtDNA and sequenced on an Illumina Miseq. SNPs in these mtDNA genomes were called with a mapping quality Phred score >30 and a minimum read depth of 10. The seven diagnostic SNPs are located in the tRNA-Cys ($n = 2$), COX3 ($n = 3$) and ND4 ($n = 2$) genes. Three 63–74 bp fragments (excluding primers) covering the seven SNPs were selected for amplification. Primers were designed from the same alignment using Primer3 in Geneious R7 (Biomatters). Forward and reverse primers for each fragment are (Fragment 1-tRNA-Cys: 5′-GTTCTCTACATAAGCC CTGG-3′, 5′-GGTCTTATTTGAACCTAAGCC-3′; Fragment 2-COX3: 5′-TTGGTTCTCTCTTCCTAAT CG-3′, 5′-TACGAAGTGTCAGTATCAGG-3′; Fragment 3-ND4: 5′-TAGCATTTGAAGCTTAACCC-3′, 5′-AGTCAGCAGGATAGGATAAG-3′).

sample	origin	SNPs 5610	5633	9918	9927	9969	10938	10965
4857	Vic	C	C	G	T	T	G	C
4862	Vic	C	C	G	T	T	G	C
4860	Vic	C	C	G	T	T	G	C
4874	Vic	C	C	G	T	T	G	C
5793	Vic	C	C	G	T	T	G	C
4881	Vic	C	C	G	T	T	G	C
4883	Vic	C	C	G	T	T	G	C
3376	Vic	C	C	G	T	T	G	C
11011	WA	C	C	G	T	T	G	C
1800	WA	C	C	G	T	T	G	C
1822	WA	C	C	G	T	T	G	C
1823	WA	C	C	G	T	T	G	C
11036	WA	C	C	G	T	T	G	C
11003	WA	C	C	G	T	T	G	C
11012	WA	C	C	G	T	T	G	C
11004	WA	C	C	G	T	T	G	C
2336	NSW	C	C	G	T	T	G	C
4854	Tas*	T	C	C	C	T	G	T
9729	Tas	T	T	C	C	C	A	T
9136	Tas	T	T	C	C	C	A	T
9092	Tas	T	T	C	C	C	A	T
9728	Tas	T	T	C	C	C	A	T
JX475454	Tas	T	T	C	C	C	A	T
JX475455	Tas	T	T	C	C	C	A	T
JX475456	Tas	T	T	C	C	C	A	T
JX475457	Tas	T	T	C	C	C	A	T
JX475458	Tas	T	T	C	C	C	A	T
JX475459	Tas	T	T	C	C	C	A	T
JX475461	Tas	T	T	C	C	C	A	T
JX475462	Tas	T	T	C	C	C	A	T
JX475463	Tas	T	T	C	C	C	A	T
JX475464	Tas	T	T	C	C	C	A	T
JX475465	Tas	T	T	C	C	C	A	T
JX475466	Tas	T	T	C	C	C	A	T
JX475467	Tas	T	T	C	C	C	A	T
C6257	?	T	T	C	C	C	A	T
C31255	?	T	T	C	C	C	A	T
C22543	?	T	T	C	C	C	A	T

Figure 2. DNA sequence variation at seven mtDNA SNPs in 17 mainland and 18 Tasmanian devils, and the three Victorian devils of unknown origin (highlighted by box). Asterisk indicates an ancient Tasmanian sample approximately 4000 years old. SNP numbers refer to nucleotide position in the devil mitochondrial genome, GenBank Accession: JX475463. Sample numbers 2336–11036 are ACAD sample numbers, JX475454–JX475467 are GenBank Accession numbers from [15].

Singleplex PCRs were performed in 25 µl volumes containing 0.5 U Platinum Taq Hifi, 1x Platinum Hifi buffer, 0.25 mM each dNTP, 2 mM $MgSO_4$, 0.25 mg ml^{-1} Rabbit Serum Albumin, 0.4 µM forward primer, 0.4 µM reverse primer and 2 µl of sample DNA. Thermocycling consisted of 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 55°C for 15 s, 68°C for 30 s, and a final extension of 68°C for 10 min. All PCRs included a negative no-template control to check for cross-sample contamination and the extraction negative control. Amplification success was determined by running 2.5 µl of product on 3.5% agarose gels stained with GelRed (Biotium, Hayward, USA). Positive amplification products were Sanger sequenced in both directions using BigDye chemistry and an Applied Biosystems 3730xl DNA Analyzer at the Australian Genome Research Facility (Adelaide, Australia).

2.3. Sequence alignment and population assignment

Sequence chromatograms were trimmed for low quality data (error probability limit = 0.05) and the reads assembled to a reference devil mitogenome (JX475463) in Geneious 7 (Biomatters) using the high sensitivity option. Ambiguities were visually inspected and consensus sequences for each of the three individuals were aligned to the original 35 Tasmanian and mainland sequences.

3. Results and discussion

We obtained 203 bp of mtDNA sequence, including the seven population-informative SNPs, for the three devil specimens recovered from Victoria. All three samples matched the contemporary Tasmanian devils at all seven SNPs (figure 2). These results suggest that the devils recovered in Victoria originated from Tasmania and do not represent a relict mainland population. The consensus of fossil evidence, suggesting that mainland devils became extinct approximately 3000 years ago [6,7], support these results. These animals may have been kept as pets or in private zoos, and they may have subsequently escaped or been released. A newspaper report [10] describing the quiet nature of the Dereel animal (C22543) captured in 1971 is consistent with a significant part of its life spent in captivity. At least two devils have been recorded as escaping from Victorian wildlife parks—one in 1939 from the Ballarat Zoological Gardens [16] and the second in 2009 from the Maru Koala Park near Grantville. There are no reports that the Ballarat animal was re-captured but a roadkill devil was found close to the Maru Koala Park a short time after it went missing [17].

Animal translocation and trade is a major contributor to the spread of disease [18] and is consequently a significant concern for the Tasmanian devil since the onset of DFTD. To guard against total species extinction, a number of disease-free, captive insurance populations have been established on mainland Australia [19]. Additionally, there have been an increasing number of calls to reintroduce devils into the mainland wild, not only as a precaution against extinction, but also as a method to suppress introduced foxes and cats that are devastating many native animal populations [11,20]. Our results emphasize that animals kept in captivity may escape or be released, which highlights the importance of identifying and controlling wildlife trade and translocation. Given the precarious nature of the Tasmanian devil in the wild, the strict quarantine regimes are important for mainland insurance populations and may become even more so if reintroduction to the mainland is considered.

Research and animal ethics. All animal samples used in this research were from museum specimens, so no animal ethics permits were required.

Data accessibility. DNA sequences for the seven diagnostic SNPs for all devil samples included in this study are available in figure 2.

Authors' contributions. J.J.A. conceived the study, collected the museum specimens, interpreted the data and revised the manuscript. L.C.W. collected and analysed the data, and drafted the manuscript.

Competing interests. The authors declare no competing interests.

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Chapter 3

High-quality fossil dates support a synchronous, late-Holocene extinction of devils and thylacines in mainland Australia

Manuscript prepared for submission

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Principal Author

Name of Principal Author (Candidate)	Lauren C White
Contribution to the Paper	Helped design the study, acquired and analysed the data and drafted the manuscript *Co-first author
Overall percentage (%)	40%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/3/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Helped design the study, analysed the data and drafted the manuscript *Co-first author		
Signature		Date	21/03/2017

Name of Co-Author	Corey J.A. Bradshaw		
Contribution to the Paper	Helped design the study, interpreted the results and helped revise the manuscript		
Signature		Date	21/03/2017

Name of Co-Author	Jeremy J. Austin		
Contribution to the Paper	Helped design the study, collected the samples, interpreted the results and helped revise the manuscript		
Signature		Date	31/3/17

High-quality fossil dates support a synchronous, late-Holocene extinction of devils and thylacines in mainland Australia

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Abstract

The last large marsupial carnivores — the Tasmanian devil (*Sarcophilis harrisii*) and thylacine (*Thylacinus cynocephalus*) — went extinct on mainland Australia during the mid-Holocene. Based on the youngest fossil dates (~3500 years before present, BP), these extinctions are often considered synchronous and driven by a common cause. However, many published devil dates have recently been rejected as unreliable, shifting the youngest mainland fossil age to 25,500 years BP and challenging the synchronous-extinction hypothesis. Here we provide 24 and 20 new ages for devils and thylacines, respectively, and collate existing, reliable radiocarbon dates by quality-filtering available records. We use this new dataset to estimate an extinction time for both species by applying an ensemble hindcasting approach based on five inferential methods. Our new data and analysis definitively support the synchronous-extinction hypothesis, estimating that the mainland devil and thylacine extinctions occurred between 3179 and 3226 years BP.

Keywords: extinction, AMS dating, thylacine, devil, Holocene

Background

During the late Pleistocene, Tasmanian devils (*Sarcophilus harrisii*) and thylacines (Tasmanian tiger or wolf, *Thylacinus cynocephalus*) were widespread over the Australian continent [1, 2]. Both species subsequently became extinct on mainland Australia, only surviving into historical times on the island of Tasmania. The thylacine was hunted to extinction after European arrival [3], while devils have suffered localised declines of more than 80% in < 20 years, due to a transmissible cancer [4]. Based on the youngest dated available fossils, both species are assumed to have become extinct on mainland Australia during the mid-Holocene (approximately 3500 years before present, BP) [5].

The cause of these extinctions is the subject of debate, with the introduction of dingoes, human intensification (i.e. development of advanced tools and population size increase), and climate change being the three main competing, but not necessarily mutually exclusive, hypotheses [3]. These three events have been postulated based on the timing of events and the likely isolation of Tasmania from their impacts.

Debate around extinction drivers have almost always assumed that both extinctions were roughly synchronous and therefore, potentially attributable to a common cause (or set of causes). However, the reliability of many fossil ages for devils across Australia has recently been questioned [6], shifting the mainland devil's youngest reliable fossil age back to 25,500 years BP and challenging the synchronous-extinction hypothesis.

The youngest fossil age of an extinct taxon is nearly always an inaccurate proxy for the final extinction date. These two dates will inevitably diverge due to incomplete sampling, taphonomic bias and uncertainty in radiometric dating [7, 8]. Many statistical models have been developed to estimate extinction time (and the associated uncertainty) using the time series of fossil ages, but their accuracy varies with the mode of extinction and sampling density over time [5].

To address these issues, we combined 44 new, high-quality ages for mainland devils and thylacines with existing data that met stringent quality requirements. We applied an ensemble-hindcasting approach [9] based on five distinct frequentist methods commonly used to infer the timing of species extinction from fossil records to calculate the mainland extinction dates for both species.

Methods

We collected < 1 g of bone or tooth from 20 thylacine and 24 devil fossils from southern mainland Australia (Figure 1, SI Table 1). Samples were radiocarbon dated at the Australian National University, the University of Waikato, or the Oxford Radiocarbon Accelerator Unit. We added these new ages to 129 existing mainland devil and 104 mainland thylacine records extracted from the *FosSahul* database (doi:10.4227/05/564E6209C4FE8) [10]. We removed all unreliable ages using a set of objective criteria based on the reliability of the dating procedure used, followed by an evaluation of the confidence in the stratigraphic relationship of the dated material to the target taxon (see full details in [6]). We calibrated all dates to calendar years (BP) using the Southern Hemisphere Calibration curve (ShCal13) from the OxCal radiocarbon calibration tool Version 4.2 (https://c14.arch.ox.ac.uk). As there is uncertainty about whether *Sarcophilus laniarius* was a separate, co-occurring species to *Sarcophilus harrisii*, or the same lineage that experienced dwarfism during the Pleistocene [11], we repeated our analyses excluding *S. laniarius* ages.

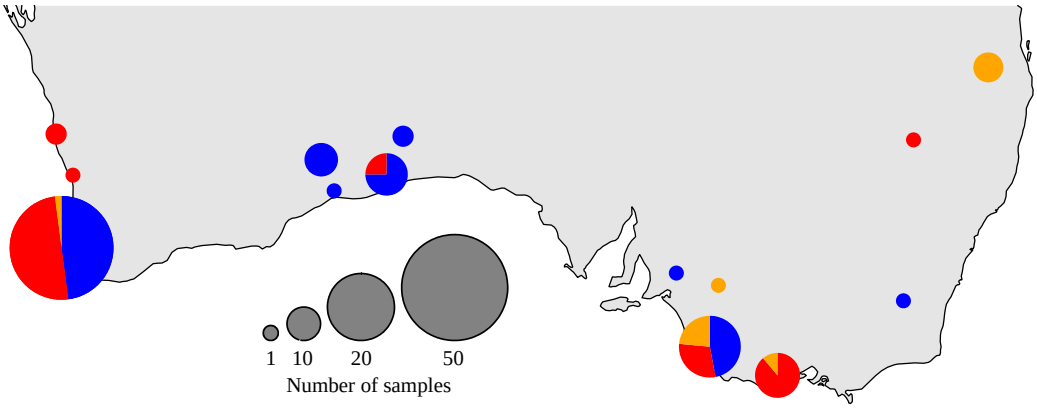

Figure 1 - Sampling location of all records used to estimate extinction times of *T. cynocephalus* (blue), *S. harrisii* (red), and *S. laniarius* (orange). We combined some closely neighbouring localities. Circle size is proportional to the number of samples.

Using the ensemble-hindcasting approach [9], we applied five different methods to infer final extinction time to the quality-filtered data: Solow's [7], Marshall's [12], McCarthy's [13], McInerny's [14], and the Gaussian-resampled inverse-weighted McInerny (GRIWM) [15]. Each method returns an extinction window (temporal confidence interval) for each species. We then calculated the distribution of cross-model agreement through time (i.e., for every year from the last 10,000 years, we calculated how many models predicted extinction for a given taxon) under the assumption that higher cross-model agreement decreases uncertainties in extinction-window estimates. Final extinction time and the associated confidence intervals (CI) for each species were calculated by bootstrapping the datasets 1000 times with replacement. We applied the ensemble-model hindcasting to each new time series to calculate the mode of each distribution and report the final extinction time as the median value of the 1000 modes with the adjusted bootstrap percentile interval as confidence intervals at 95% (see full description of the approach in SI).

Results and Discussion

The 44 new radiocarbon ages for mainland devils and thylacines (SI Table 1), include the youngest, reliably dated samples for each species (devil: 3245 ± 62 and thylacine: 3290 ± 49 years BP). All our new ages passed quality filtering, but only 31 of the 129 previous devil (24.0%) and 27 of 104 thylacine (26.0%) ages in the *FosSahul* database [10] met the minimum reliability criteria (A*- or A-rated only) [10]. Adding these records to the new dates produced final, high-quality datasets of 56 devil and 48 thylacine ages. Excluding *S. laniarius* from the devil dataset left 45 reliable ages.

Continent-wide multi-averaging produced mainland extinction estimates (T_{ext} in Figure 2) of 3180 years BP (CI: 3179-3182) for devils, and 3225 years BP (CI: 3223-3226) for thylacines (SI Table 2). Removing *S. laniarius* barely modified the estimate for devils (ΔT_{ext} = 2 years). The relevance of multi-averaging estimates could be questioned because it relies on results from a collection of statistical methods that might be inappropriate under some conditions [5, 16, 17]. With this in mind, we additionally took a second approach in which the most appropriate model was chosen using a published decision tree. This analysis found that GRIWM was the most appropriate method as a function of statistical characteristics of the dataset [5]. The results from GRIWM supported the outputs of the multi-averaging (see full analysis in electronic supplementary materials section 'Model selection'), producing similar results.

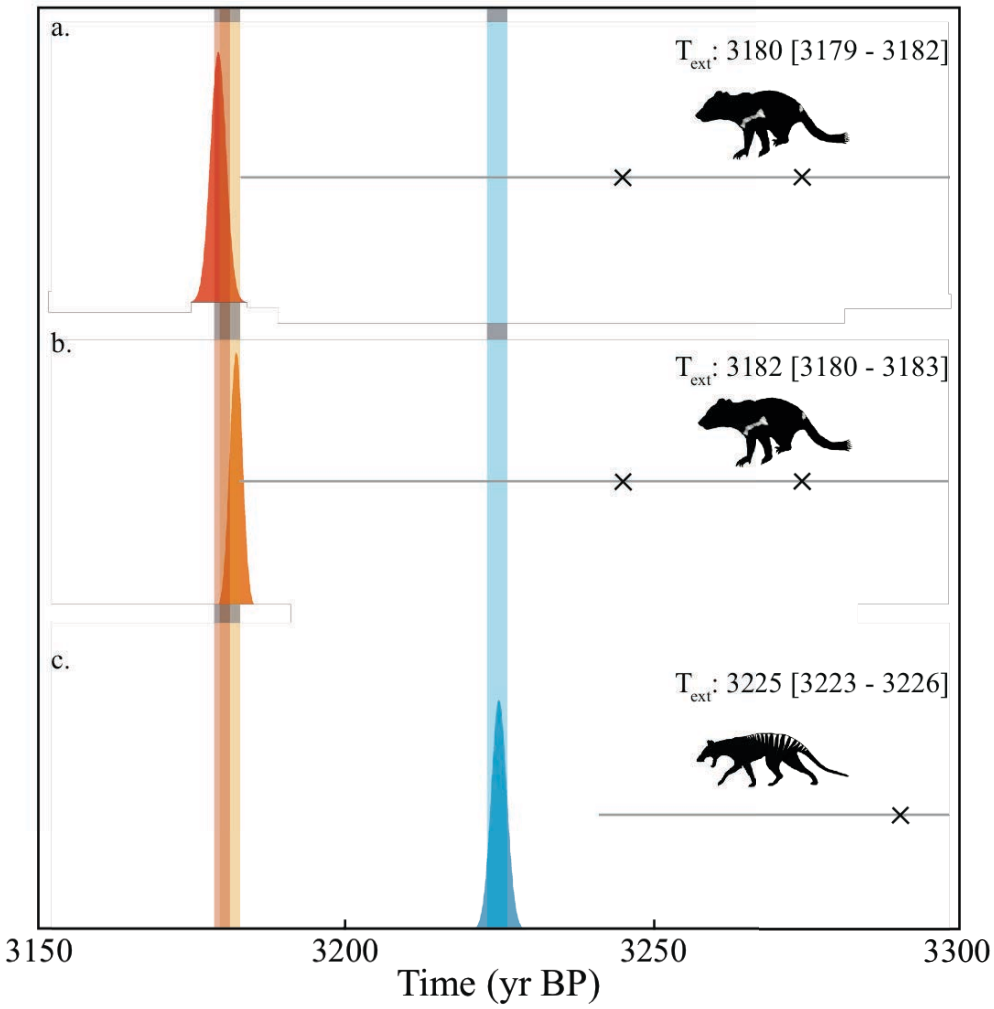

Figure 2 – Estimated extinction windows (vertical columns) as a function of the ensemble-hindcasting approach for (a.) *Sarcophilus* (light red/brown), (b.) *Sarcophilus* excluding *S. laniarius* (light orange/brown), and (c.) *Thylacinus* (light blue) on mainland Australia. Vertical columns are calculated as the 95% confidence interval of the distribution (dark red, orange and blue) of the 1000 modes of bootstrapped ensemble hindcasting inferences. Crosses indicate fossil ages (arbitrarily limited between 3150 and 3300 years BP for the clarity of the figure) and the grey line denotes the associated standard deviation (σ) uncertainties. The brown vertical bar indicates the period of extinction overlap between the red and orange vertical bar.

Our youngest reliable age for mainland devils contrasts the most recent estimate based on high-quality ages at 25,500 years BP [6]. As such, the addition of 24 new dates changes the reliable persistence timeline for this species by approximately 22,000 years. Younger dates assessed by Rodríguez-Rey *et al*. [6] were mostly rejected based on inappropriate pre-treatment protocols and/or unsuitable materials used, highlighting the importance of choice in dating method.

Our model-averaging constrains the reliable dates of mainland devil and thylacine extinction to within a short (< 50-year) period, between 3179 and 3226 years BP, that is consistent with a scenario of synchronous extinction. Synchronous extinctions have been used on other continents and at different time points as evidence for large-scale, common extinction drivers [9, 18-20]. For example, analysis of the extinction chronology in North America's Pleistocene mammals suggested a single event wiped out up to 35 genera across the continent over a 2000-year period [20]. Extending this concept to derive the most likely cause of extinctions, Cooper *et al*. [18] examined multiple waves of synchronous extinctions across the Holarctic and found them coincident with climate warming events that likely exacerbated declines arising from human hunting. Conversely, the concurrent extinction of Australian megafauna during the Pleistocene seems to be independent of continental-scale climate change, potentially indicating a dominant human role [9, 21].

Under the assumption that the mainland devil and thylacine extinctions were coincident, several studies have explored possible causes. For example, as the dingo arrived in Australia ~4000 years BP and never reached Tasmania, dingoes have been suspected of driving the mainland extinctions of devils and thylacines [22]. Johnson and Wroe [23] suggested that human innovation in hunting technology, and more intensive use of resources could also have led to the mainland extinctions. Prowse *et al.* [3] used a modelling approach to conclude that

human intensification, followed by climate change, were the most likley candidate determinants.

Other studies have avoided the assumption of synchronous extinctions by focusing on devils or thylacines separately. Letnic *et al*. [24] used morphological analyses to conclude that direct killing of thylacines by dingoes was biologically feasible and could therefore have contributed to their demise. Similarly, Brown [25] argued that climate instability associated with the onset of El Niño-Southern Oscillation (ENSO) events could have affected mainland devils, but not thylacines.

Our estimated extinction window for mainland devils and thylacines is similar to the assumed, but until now unvalidated, extinction time in most previous studies, and therefore does not challenge any aforementioned arguments, nor do our results exclude the possibility of separate or multiple causes of these extinctions. However, by supporting the assumption of synchronous extinctions with reliably dated fossil specimens, and taking into account that the youngest fossil age is an inaccurate proxy for the true extinction time for the first time, our analyses provide a strong and defendable basis on which further research can build. Our understanding of these extinctions will become more complete as more palaeoclimatic, palaeoecological, and archaeological data are used to uncover the biogeographic histories of these species.

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Competing Interests

The authors declare no competing interests

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Authors' Contributions

LCW helped design the study, acquired and analysed the data and drafted the manuscript; FS helped design the study, analysed the data and drafted the manuscript; CJAB helped design the study, interpreted the results and helped revise the manuscript; JJA helped design the study, collected the samples, interpreted the results and helped revise the manuscript. All authors gave final approval for publication.

Data Accessibility

All new radiocarbon dates are available from the AEKOS Data Portal (doi:10.4227/05/57BA9BE3ABC2A) and have been added to the FosSahul database (doi:10.4227/05/564E6209C4FE8)

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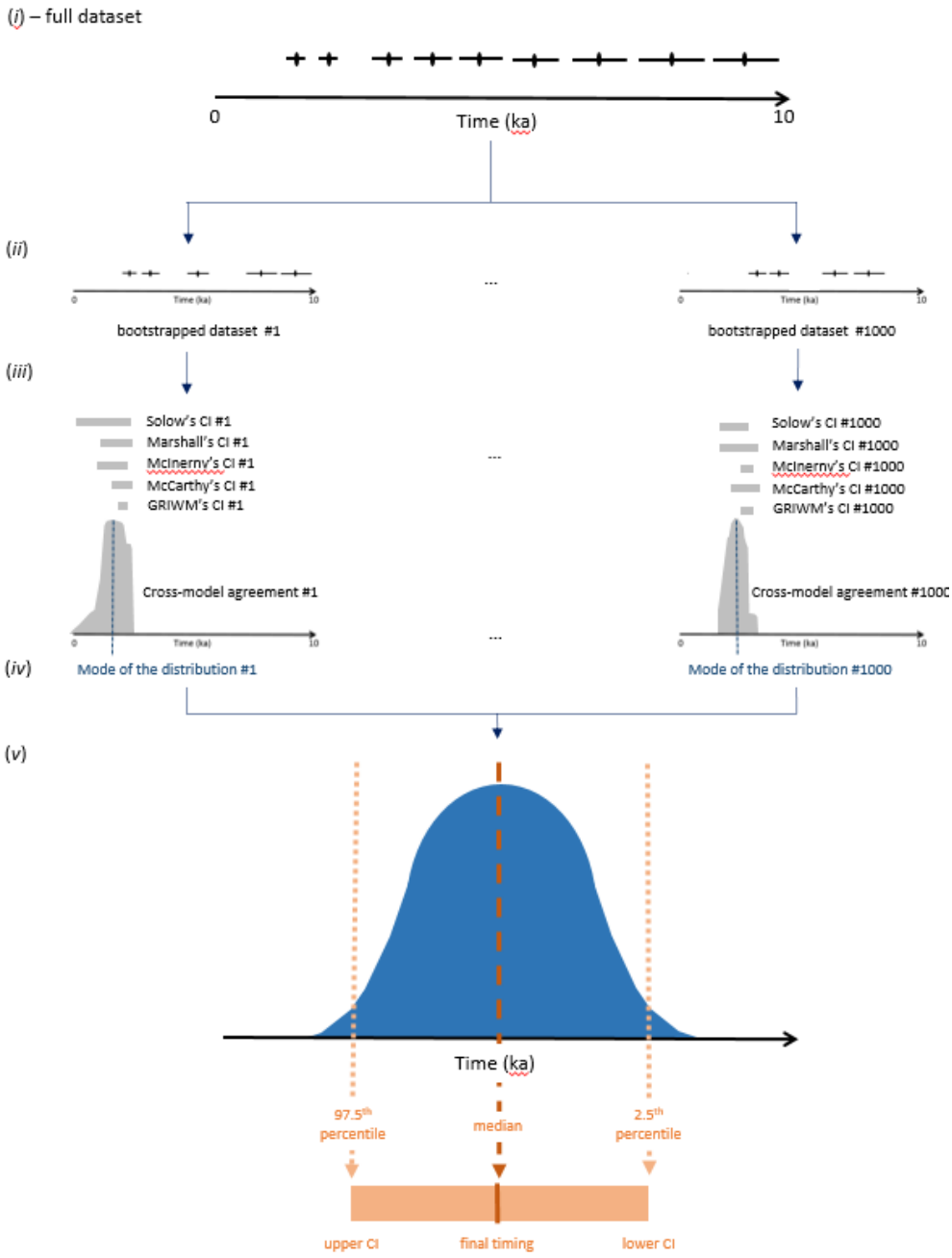
Supplementary Information and Methods

Model ensemble-hindcasting approach

Our approach computes the extinction window for each taxon as a function of five established frequentist methods originally developed to estimate the time gap between the last dated fossil and the final extinction date: (1) McCarthy's (1998) [1], (2) Marshall's (1997) [2], (3) Solow's (2006) [3], (4) McInerny's (2006) [4] methods, and (5) the Gaussian-Resampled Inverse-Weighted Method (GRIWM) [5].

Each method returns an extinction window (temporal confidence interval) for each taxon assuming different underlying statistical properties. For example, Solow's and McInerny's methods assume a uniform probability of record occurrence. Other methods relax this assumption, either by integrating some temporal variation (i.e., a 'recovery function', see [2] and [1], calculated here as a function of a probability of sampling fitted to each given time series following the approach described in [6]) or by constraining the probability of sampling (GRIWM) [5], to assume that the probability of discovering fossil records decreases near the terminal date [7]; however, the assumption of independence among records is still required. Finally, GRIWM and Solow's models are the only approaches we tested here that take into account the uncertainties in record dates, allowing extinction preceding the last record if the error on that estimate is high, but because Solow's assumes constant dating uncertainties across samples, GRIWM assumes variation in these uncertainties by 10,000 (or more) resamples of the standard deviation of each date from a Gaussian distribution [5].



SI Figure 1 – Flow chart of the model ensemble-hindcasting approach.

From these five method-specific outputs we built an estimate of timing of extinction (along with a confidence interval) for each taxon by calculating a window of cross-model agreement through time applying the assumption that higher cross-model agreement decreases uncertainties in extinction-window estimates (SI Figure 1). The confidence interval of the timing of extinction is calculated for each taxon (*i*) by taking 1000 bootstrap re-samples of

the dataset (i.e., the time series of fossil ages); (*ii*) we applied the six methods of estimating extinction timing to each of the 1000 bootstrapped resampled datasets, and (*iii*) calculated for every year over the period 10,000 to 0 years ago how many models predicted extinction for a given taxon; (*iv*) we used the mode of the distribution of cross model agreement values calculated in *iii* as an estimate of the timing of extinction for each bootstrapped dataset, resulting in 1000 estimates of timing of extinction for each taxon; (*v*) we reported the median of these 1000 estimates as the final estimated extinction time along with the 95% confidence interval determined by the 2.5^{th} and 97.5^{th} percentiles of the 1000 values. To avoid any potential bias due to the skewness of the distribution of the 1000 estimates in (*iii*), we used the bias-corrected and accelerated bootstrap approach [8] to calculate the 95% confidence interval.

Model selection

Saltré *et al*. [9] provided a general guide (model-selection key) for choosing the most appropriate model among eight frequentist approaches (including the five methods we applied here) for a given series of dated fossils. Identifying the appropriateness of the models depends largely on how they treat both the probability of record occurrence and the uncertainties in record dates. We characterized time series of dated records as a function of five times-series characteristics: (1) number of records ($\boldsymbol{n}$); (2) average and (3) variance of the interval between successive records ($\bar{\boldsymbol{\imath}}$ and $\boldsymbol{\sigma^2 i}$, respectively); and (4) average and (5) variance of dating error ($\bar{\boldsymbol{\epsilon}}$ and $\boldsymbol{\sigma^2 \epsilon}$, respectively) covering the time-series range. We first calculated the characteristics for both *Sarcophilus* and *Thylacinus* (SI Table 3), and then reported these characteristic in the model-selection key (Figure 4 of ref [9]) to identify the most appropriate method for each genus. Overall, we recommend using either Solow's, McInerny's or GRIWM for both genera (SI Table 2). Based on $\bar{\boldsymbol{\imath}}$**,** the model-selection key

suggested using Solow's, McInerny's and GRIWM. While $\boldsymbol{\sigma^2 i}$ prescribes mainly Solow's and McInerny's models, the high $\bar{\epsilon}$ is probably the cause of their lower accuracy; this promotes GRIWM because of its slightly narrower confidence intervals and the more realistic way in which it takes into account the radiometric uncertainties of each age. Regardless, Solow's, McInerny's and GRIWM outputs are close ($\Delta_{\text{median (}\textit{Sarcophilus}\text{)}}$ = 63 years; $\Delta_{\text{median (}\textit{Thylacinus}\text{)}}$ = 42 years) and match the estimates using ensemble-model hindcasting. Excluding *Sarcophilus laniarius* from the dataset slightly modified the summary characteristics, but still indicated that GRIWM is the most appropriate method.

SI Table 1: Details of specimens newly radiocarbon dated for this study

Museum ID	Museum	Latitude	Longitude	State	Genus	Species	AgeID	Age (calBP)	Sd
76.4.40	Western Australian Museum	-34.2747	115.0983	WA	Thylacinus	cynocephalus	ANU-44733	3290	49
MB103/AD135	TREND, Curtin University	-31.6583	128.0799	WA	Thylacinus	cynocephalus	ANU-43733	3372	51
04.2.280	Western Australian Museum	-34.322	115.142	WA	Thylacinus	cynocephalus	ANU-43715	3498	44
73.7.132	Western Australian Museum	-34.322	115.142	WA	Thylacinus	cynocephalus	ANU-43732	3737	59
75.9.15	Western Australian Museum	-30.222	128.757	WA	Thylacinus	cynocephalus	ANU-43736	3944	54
75.9.17	Western Australian Museum	-30.222	128.757	WA	Thylacinus	cynocephalus	ANU-43735	3972	58
64.8.1	Western Australian Museum	-32.0441	126.0376	WA	Thylacinus	cynocephalus	ANU-43739	4261	76
69.12.13	Western Australian Museum	-34.2001	115.08	WA	Thylacinus	cynocephalus	ANU-43728	4436	63
F6355	Western Australian Museum	-31.7039	127.735	WA	Thylacinus	cynocephalus	ANU-43724	4469	50
04.2.249	Western Australian Museum	-34.2001	115.08	WA	Thylacinus	cynocephalus	ANU-43716	4976	87
04.2.463	Western Australian Museum	-31.6621	128.7821	WA	Thylacinus	cynocephalus	ANU-43719	4976	87
04.2.359	Western Australian Museum	-31.005	125.528	WA	Thylacinus	cynocephalus	ANU-43714	5163	82
04.2.347	Western Australian Museum	-31.005	125.528	WA	Thylacinus	cynocephalus	ANU-43717	5167	81
04.2.345	Western Australian Museum	-31.005	125.528	WA	Thylacinus	cynocephalus	ANU-43718	5181	87
76.10.440	Western Australian Museum	-34.2001	115.08	WA	Thylacinus	cynocephalus	ANU-43721	5519	51
04.2.360	Western Australian Museum	-31.005	125.528	WA	Thylacinus	cynocephalus	ANU-43720	5828	54
F6356	Western Australian Museum	-31.7039	127.735	WA	Thylacinus	cynocephalus	ANU-43738	6175	75
69.12.3	Western Australian Museum	-34.2001	115.08	WA	Thylacinus	cynocephalus	ANU-43729	6333	41
69.9.11	Western Australian Museum	-31.005	125.528	WA	Thylacinus	cynocephalus	ANU-43730	7407	49
CM15266	Australian National Wildlife Collection	-35.7167	148.5	NSW	Thylacinus	cynocephalus	Wk-36184	8108	48
Unregistered	Museum Victoria	-37.989627	140.9905	VIC	Sarcophilus	harrisii	ANU-43706	3245	62
Unregistered	Museum Victoria	-37.989627	140.9905	VIC	Sarcophilus	harrisii	OxA-29918	3274	51

Unregistered	Museum Victoria	-37.989627	140.9905	VIC	Sarcophilus	harrisii	OxA-29921	3390	42
Unregistered	Museum Victoria	-37.989627	140.9905	VIC	Sarcophilus	harrisii	OxA-29915	3402	36
04.3.278	Western Australian Museum	NA	NA	WA	Sarcophilus	harrisii	ANU-43713	3493	45
C30654	Australian National Wildlife Collection	-30.3552	148.8984	NSW	Sarcophilus	harrisii	Wk-36185	3516	41
88.10.1.b	Western Australian Museum	-31.7378	127.7888	WA	Sarcophilus	harrisii	OxA-29910	3696	61
P157490	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	OxA-29917	3782	52
Unregistered	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	ANU-43709	4285	73
Unregistered	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	ANU-43707	4292	72
F6328	Western Australian Museum	-31.7039	127.735	WA	Sarcophilus	harrisii	ANU-43711	4599	100
Unregistered	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	OxA-29919	4619	98
71.10.209	Western Australian Museum	-34.2001	115.08	WA	Sarcophilus	harrisii	ANU-44730	4627	98
Unregistered	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	ANU-43710	5178	85
Unregistered	Museum Victoria	-37.989627	140.9905	VIC	Sarcophilus	harrisii	OxA-29916	6332	39
P173273	Museum Victoria	-38.349	142.3573	VIC	Sarcophilus	harrisii	OxA-29922	6393	47
P157487	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	OxA-29913	6776	53
88.10.1.b	Western Australian Museum	-33.6421	115.0358	WA	Sarcophilus	harrisii	OxA-29912	7146	73
P21377	Museum Victoria	NA	NA	VIC	Sarcophilus	harrisii	OxA-29914	7756	53
P157512	Museum Victoria	-37.8406	142.8803	VIC	Sarcophilus	harrisii	OxA-29920	9167	78
68.7.36	Western Australian Museum	-31.521	115.731	WA	Sarcophilus	harrisii	OxA-29908	11753	128
73.11.379	Western Australian Museum	-30.15	115.0666	WA	Sarcophilus	harrisii	OxA-29907	16238	115
75.5.707	Western Australian Museum	-30.15	115.0666	WA	Sarcophilus	harrisii	OxA-29909	17242	123
68.3.245	Western Australian Museum	-34.303	115.297	WA	Sarcophilus	harrisii	OxA-29906	17628	114

SI Table 2 – Final extinction timing along with the confidence interval (CI) inferred from five statistical methods (Marshall, McCarthy, Solow, McInerney, GRIWM) for *Sarcophilus* spp., *Sarcophilus* excluding *Sarcophilus laniarius* (i.e., no *S. laniarius*), and *Thylacinus*. Model-averaging results for each group are reported as the confidence interval at the 97.5th and 2.5th percentiles of the mode of the distribution of 1000 modes of distribution of model-average agreements calculated on the same 1000 time series subsampled by bootstrapping with replacement. Marshall: [2]; McCarthy: [1]; Solow: [3]; McInerny: [4]; GRIWM: [5].

Genus	**Timing of extinction (BP)**					
	Marshall	*McCarthy*	*Solow*	*McInerny*	*GRIWM*	*Model-averaged*
Sarcophilus spp.	3245 [3243 – 3245]	3244 [3243 – 3244]	3243 [3064 – 3245]	3205 [3073 – 3245]	3179 [3131 – 3224]	3180 [3179-3182]
Sarcophilus (no *S. laniarius*)	3245 [3243 – 3245]	3244 [3243 – 3244]	3245 [3086 – 3245]	3195 [3030 – 3241]	3185 [3137 – 3225]	3182 [3180-3183]
Thylacinus	3290 [3288 – 3290]	3289 [3288 – 3289]	3269 [3153 – 3290]	3243 [3086 – 3287]	3227 [3170 – 3281]	3225 [3223-3226]

SI Table 3 - Coefficient of variation (CV) for the set of summary characteristics ($\boldsymbol{n}$, $\bar{\boldsymbol{\iota}}$, $\boldsymbol{\sigma^2 i}$, $\bar{\boldsymbol{\epsilon}}$, $\boldsymbol{\sigma^2 \epsilon}$) for the time series

Genus	$\boldsymbol{n}$	$\bar{\boldsymbol{\iota}}$	$\boldsymbol{\sigma^2 i}$	$\bar{\boldsymbol{\epsilon}}$	$\boldsymbol{\sigma^2 \epsilon}$
Sarcophilus spp.	1.14	0.04	0.02	4253	569
Sarcophilus (no *S. laniarius*)	0.88	0.05	0.02	768	71
Thylacinus	0.96	0.04	0.01	1086	60

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Chapter 4

Ancient mitochondrial genomes reveal the demographic history and phylogeography of the extinct, enigmatic thylacine (*Thylacinus cynocephalus*)

Manuscript accepted for publication at *Journal of Biogeography*

Statement of Authorship

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Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Lauren C White
Contribution to the Paper	Acquired and analysed the data, and drafted the manuscript
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/3/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Designed the study, collected samples, interpreted the results and helped revise the manuscript.
Signature	Date 31/3/17

ORIGINAL ARTICLE

Ancient mitochondrial genomes reveal the demographic history and phylogeography of the extinct, enigmatic thylacine (*Thylacinus cynocephalus*)

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ABSTRACT

Aim: The Tasmanian tiger, or thylacine, is an infamous example of a recent human-mediated extinction. Confined to the island of Tasmania in historical times, thylacines were hunted to extinction less than 150 years after European arrival. Thylacines were also once widespread across the Australian mainland, but became extinct there ~3,200 years before present (BP). Very little is known about thylacine biology and population history; the cause of the thylacines extirpation from the mainland is still debated and the reasons for its survival in Tasmania into the 20th century are unclear. In this study, we aimed to investigate the thylacine's phylogeography and demographic history leading up to their extinction on both the mainland and Tasmania to gain insight into this enigmatic species.

Location: Southern Australia

Methods: We generated 51 new thylacine mitochondrial DNA (mtDNA) genome sequences from sub-fossil remains and historical museum specimens, and analysed them to reconstruct the species' phylogeography and demographic history.

Results: We found evidence that thylacines had contracted into separate eastern and western populations prior to the Last Glacial Maximum (~25,000 years BP), and that the ancient western population was larger and more genetically diverse than the historical Tasmanian population. At the time of European arrival in ~1800 CE, Tasmanian thylacines had limited mtDNA diversity, possibly resulting from a bottleneck event during the late Pleistocene or Holocene, but we find some indication that the population was expanding during the late Holocene.

Main Conclusions: The timing of this putative expansion is in concert with an El Niño Southern Oscillation (ENSO) associated climate event, suggesting that climate change had an influence on thylacine population dynamics. Given that ENSO effects are known to have been more severe on mainland Australia, we suggest that climate change, in synergy with other drivers, is likely to have contributed to the thylacine mainland extinction.

KEYWORDS: aDNA, ancient DNA, Australia, mitogenomes, phylogenetics, Tasmanian tiger, Tasmanian wolf

INTRODUCTION

The Tasmanian tiger, or thylacine (*Thylacinus cynocephalus*; Harris, 1808), is a marsupial carnivore infamous for its recent, human-mediated extinction. At the time of European arrival in Australia in the late 1700s, the species was found only on the island of Tasmania. It became extinct less than 150 years later, likely due to hunting encouraged by bounty schemes initiated because of its perceived impact on introduced livestock (Owen, 2003). The Tasmanian devil (*Sarcophilus harrisii*), similarly confined to Tasmania, then inherited the title of the largest extant marsupial carnivore. Both species were also once widespread across mainland Australia before declining to extinction there approximately 3,200 years before present (BP; Johnson, 2006).

The driver(s) of the late-Holocene mainland extinctions is still debated. Changes in climate, human intensification (i.e. development of advanced tools and population size increase), and the introduction of the dingo are the three main hypothesised causes (Prowse *et al.*, 2013). The basis for these hypotheses is the timing of the changes/events and the isolation

of Tasmania from their likely impacts. For example, the dingo (*Canis lupus dingo*) - a potential predator and competitor of the thylacine and devil - was introduced to mainland Australia ~5,000 years BP but never reached Tasmania as rising sea levels had flooded Bass Strait thousands of years earlier (~14,000 years BP; Corbett, 1995). Similarly, human population size and hunting impacts increased on mainland Australia during the Holocene while this trend was markedly absent in Tasmania, where population size remained low (Johnson & Wroe, 2003). Finally, following the relatively wet and stable period of the Holocene optimum (~8,000-5,000 years BP), a strengthening of the El Niño Southern Oscillation (ENSO) caused a shift in Australia's climate towards a drier, more drought-prone system (Donders *et al*., 2008).

The effects of late-Holocene ENSO activity are assumed to have had a lesser influence on Tasmania — due to its maritime climate and more consistent rainfall (Donders *et al.,* 2007; Donders *et al.,* 2008, Rees *et al.,* 2015) —and hence, had a reduced impact on Tasmanian devils and thylacines (Brown, 2006). However, a recent genetic study found evidence for a bottleneck in the Tasmanian devil population that is coincident with a peak in ENSO activity and the mainland population's extinction approximately 3,200 years BP (Brüniche-Olsen *et al.,* 2014). The absence of other possible drivers in Tasmania suggests that shifts in climate may have initiated the decline of devil populations in Tasmania and the mainland. The combined pressure of climate change, dingoes and/or human intensification on the mainland may have led to the devil's extinction there. Given that climate change effects are expected to have been greater on the mainland than on Tasmania, Brüniche-Olsen *et al*. (2014) suggest that climate change may have been underestimated as a driver of the late-Holocene extinctions.

The late-Holocene bottleneck in Tasmanian devils resulted in the observed low genetic diversity in the population today (Jones *et al.,* 2004). Similar patterns have been observed in

historic Tasmanian thylacines (Menzies *et al.*, 2012), suggesting a common population history in the two species. However, due to the rapidity of the decline of thylacines we know very little about their biology and population history. Additionally, lack of temporal sampling has thus far prohibited analyses of historical demography and range-wide phylogeographic structure in thylacines. We obtained 51 new thylacine mitochondrial genome sequences, including the first sequences from ancient samples from both Tasmania and the mainland. We used these data to investigate the demographic history of thylacines and test the hypothesis that they underwent a similar population decline to the Tasmanian devils during the late-Holocene.

MATERIALS AND METHODS

Sample Collection, DNA Extraction and Radiocarbon Dating

We collected < 2 g of bone, tooth or dried tissue from 81 mainland and Tasmanian thylacines held in various museums (Figure 1, also see Appendix S1, Table S1.1 in supporting information) using a Dremel tool (Racine, WI, USA) fitted with Dremel cut-off wheel #409 (for bone samples) or sterilised scalpel blades (for tissue samples).

We controlled for contamination of the subfossil and historic museum samples with contemporary DNA by conducting all pre–PCR work in a dedicated and physically separate clean–room DNA facility at the Australian Centre for Ancient DNA, University of Adelaide. Laboratory protocols included: the use of still-air glove boxes fitted with internal ultraviolet (UV) lights for extraction of DNA and set-up of PCR; regular decontamination of all work areas and equipment with sodium hypochlorite; personal protective equipment, including disposable laboratory gowns, face masks, face shields, shoe covers and double-gloving; and

strict one-way movement of samples.

DNA extraction was performed using the protocol described in Brotherton *et al*. (2013) with some small changes (see Appendix S2). Subfossil samples with enough material left after DNA extraction (n=19) were submitted for radiocarbon dating at the Australian National University or the University of Waikato. We calibrated all ^{14}C dates to calendar years (BP) using the Southern Hemisphere Calibration curve (ShCal13) from the OxCal radiocarbon calibration tool Version 4.2 (https://c14.arch.ox.ac.uk). Historical museum samples without a known collection date (n=15) were assigned an age of 120 years BP as an intermediate age between the death of the last known thylacine in 1936 and establishment of the Tasmanian Museum and Art Gallery in 1843, from which many of the historic samples were sourced. We define ancient samples as those that are > 600 years old and historical samples as those that are < 600 years old or that were recently deceased at the time of collection if no date was recorded.

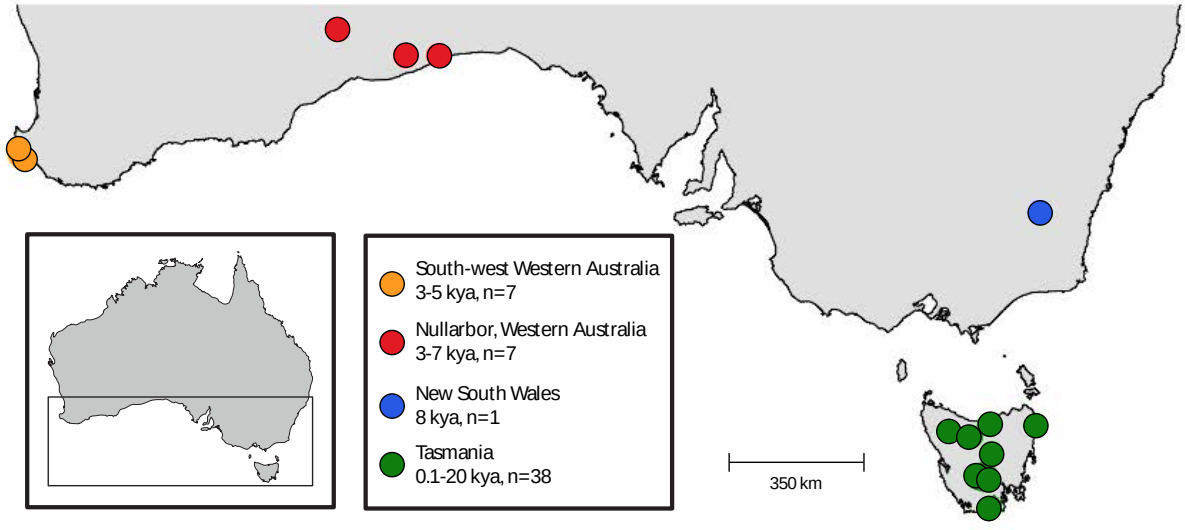

Figure 1. Sample locations of sequenced thylacine individuals coloured by broad geographic area. We combined some closely neighbouring localities. Seventeen of the 38 Tasmanian samples did not have locations recorded and are therefore not represented on the map.

Library Preparation and Hybridisation Enrichment

We built double-stranded Illumina libraries from 20 µL of each DNA extract and extraction blank control following the protocol from Meyer & Kircher (2010). We used custom adapters that featured internal barcode sequences to allow multiplexing of individuals and in-silico de-multiplexing downstream. Every batch of libraries prepared included a library blank control.

Commercially synthesised biotinylated 80-mer RNA baits (MYcroarray, MI, USA) were used to enrich the target libraries for thylacine mitochondrial DNA. Baits were designed as part of the commercial service using published thylacine mitochondrial sequences from Miller *et al*. (2009). A second set of baits was designed to include the mitochondrial genome sequence of a mainland thylacine produced using the first set of baits. We chose to exclude the control region from the second set of baits because the large amounts of repetitive DNA in that region had resulted in low mapping quality. One round of hybridisation capture was performed per the manufacturer's protocol (MYbaits, v2 manual) with modifications (see Appendix S2).

All enriched libraries were quality tested using the Tapestation 2200 (Agilent Technologies, Santa Clara, USA) and sequenced in 2x150 (i.e. paired-end) reactions on Illumina NextSeq and MiSeq machines at the Australian Genome Research Facility, Adelaide.

Sequence Processing and Mitochondrial Genome Assembly

Raw reads were de-multiplexed and internal barcodes removed using sabre (https://github.com/najoshi/sabre) before being processed and mapped to a thylacine mitochondrial reference sequence (GenBank Accession: NC011944) using the PALEOMIX 1.1 pipeline (Schubert *et al*., 2014). Briefly, we removed adapter contamination using the default

settings in ADAPTERREMOVAL 2.1 (Lindgreen, 2012) except using a minimal read length of 25 bp. Mapping was performed using BWA 0.7.7 (Li & Durbin, 2009), disabling the seed and relaxing the edit distance (option –n=0.01) as suggested by Schubert *et al.* (2012). Separate sequencing runs of the same libraries were combined before PCR duplicates were removed using SAMTOOLS 0.1.18 (Li *et al*., 2009) and MARKDUPLICATES from the Picard package (http://broadinstitute.github.io/picard/). MAPDAMAGE2 (Jónsson *et al.*, 2013), implemented in PALEOMIX, was used to demonstrate damage patterns consistent with ancient DNA template by modeling post-mortem DNA damage from patterns of nucleotide misincorporations for each library.

Finally, all alignments were visually inspected in GENEIOUS 10.0.2 and consensus sequences were called for all positions where > 60% of the sequences agreed and read depth was at least three. Where there was no > 60% majority, bases were called as the appropriate IUPAC ambiguity symbol. Regions with insufficient read depth were coded as N.

We aligned all consensus sequences and two publically available Tasmanian thylacine mitogenome sequences (GenBank Accession: NC011944 and FJ515781; Miller *et al*., 2009) using MAFFT (Katoh *et al*., 2002) as implemented in GENEIOUS. We chose to trim the control region from the alignment because of low coverage and poor mapping quality.

Descriptive Statistics

Descriptive statistics (haplotype diversity H_d, nucleotide diversity π, number of segregating sites S, and the average number of segregating sites between individuals k) were calculated on samples grouped by geography and temporal period using DNASP 5.1 (Librado & Rozas, 2009). The single NSW sample was excluded from this analysis because it was the sole representative of that geographic area. Undated ancient samples were also excluded, as they could not be accurately placed into a temporal period. For comparison, we calculated the

same statistics on a sample (n=13) of contemporary devil mitochondrial genomes (Genbank Accession: JX475454-67; Miller *et al*., 2011) which were modified to also exclude the control region.

DNASP was also used to test for demographic changes in the historical Tasmanian thylacine samples using Tajima's D (Tajima, 1989), and Fu and Li's estimators D* and F* (Fu & Li, 1993). The significance of the demographic estimators was obtained by examining the null distribution of 5,000 coalescent simulations of these statistics. Demographic estimator analysis was restricted to the historical Tasmanian samples to avoid effects of heterochrony (Depaulis *et al*., 2009).

To further test for evidence of population expansion in the historical Tasmanian samples we generated a pairwise mismatch distribution (Rogers & Harpending, 1992) on the data in DNASP. The number of observed differences between pairs of mitochondrial genomes was compared to the expected distribution of differences under specified demographic models (i.e. constant population size or population growth). By using τ, the mode of the observed mismatch distribution, and the mean mutation rate inferred for the Tasmanian population using BEAST (see below) we estimated the time of expansion by the relationship $t = \tau/2u$, in which t is the time of expansion and u is the cumulative (across sequence) probability of substitution. To this result we added the average age of all the historic thylacines (165 years), to calculate the time of expansion in years BP. The calculation was carried out using the online tool provided by Schenekar & Weiss (2011).

Phylogenetic Analysis

The program POPART (Leigh & Bryant, 2015) was used to construct a TCS haplotype network from the alignment of all sequenced individuals and the two publically available

sequences, including samples with unknown ages (n=53). Sites with more than 5% missing data were masked (Leigh & Bryant, 2015).

We constructed a time-scaled phylogenetic tree in BEAST 2.4.1 (Bouckaert *et al.*, 2014) using the same alignment. We used the mean calibrated radiocarbon date and the known or estimated collection dates for historic specimens as calibration points (Bouckaert *et al.*, 2014). When we included our ancient samples without radiocarbon/collection dates in order to estimate their ages (Shapiro *et al.*, 2011), our BEAST analyses failed to converge. Consequently, we excluded our ancient samples with unknown ages from the final BEAST analysis (n=44). The coalescent extended Bayesian skyline model (Heled & Drummond, 2008) with a relaxed lognormal clock was used as it was preferred to the constant population size coalescent when tested using the modified Akaike information criterion (AICM) in TRACER 1.6 (Table S2.2, Baele *et al.*, 2012). Despite the intra-species nature of the data, our relaxed lognormal clock analysis rejected the use of a global clock (i.e. the posterior estimates for the coefficient of variation were non zero; Drummond & Bouckaert, 2015).

An appropriate partitioning scheme for phylogenetic analysis was determined using the program PARTITIONFINDER 1.1.1 (Lanfear *et al.*, 2012). We used an input of 43 regions: first, second, and third codon positions of each mitochondrial protein-coding gene; non-coding regions; 12s rRNA; 16s rRNA; and concatenated tRNAs (Table S2.3). The optimum partitioning scheme was chosen based on the Bayesian Information Criterion. The BEAST Markov chain Monte Carlo (MCMC) was run twice with different seed values for 30 million generations sampling every 1,000 generations. All parameters showed convergence and sufficient sampling in both runs (indicated by effective sampling sizes above 200) when inspected in TRACER 1.6, with the first 10% of samples discarded as burn-in (Rambaut *et al.*, 2014). A maximum clade credibility (MCC) tree was annotated in TREEANNOTATOR 2.4.1 and visualised in FIGTREE 1.4.2 (Rambaut, 2007).

A date randomisation test was conducted to check whether the temporal signal from the radiocarbon dates associated with ancient and historic sequences were sufficient to calibrate the analysis (Ho *et al*., 2011). This test randomises all dates and determines whether the 95% high posterior density (HPD) intervals of the mean rates estimated from the date-randomised datasets include the mean rate estimated from the original data set (Figure S2.1-2). In addition, a 'leave-one-out-cross-validation' (LOOCV) test was performed to test for bias and error in the sequences and associated dates (Shapiro *et al*., 2011). In particular, we wished to test whether the assumed date of 120 years BP was appropriate for historic samples without specific dates attached to them (Figure S2.3). Input .xml files for the date randomisation and LOOCV tests were generated using the R package 'TipDatingBeast' (Rieux & Khatchikian, 2016).

Inferences of Demographic History

We used the extended Bayesian skyline model implemented in BEAST 2.4.1, with prior and MCMC settings as above, to estimate the demographic history of the Tasmanian thylacine population. We restricted this analysis to the Tasmanian population as the phylogenetic analysis of the whole dataset revealed significant structure and the date randomisation test showed insufficient temporal information among the WA samples alone (Figure S2.2). As above, the analysis was run twice and in both runs all parameters showed convergence and sufficient sampling, with the first 10% of samples discarded as burn-in.

We also inferred the thylacines demographic history from the dated mitochondrial sequences using Approximate Bayesian Computation (ABC) as implemented in DIYABC 2.1.0 (Cornuet *et al*., 2014). We tested six scenarios that represent an ancestral divergence followed by different combinations of bottlenecks and expansions in two geographically separated groups (Tasmania vs. Western Australia; Figure 2). The single NSW sample was

also excluded from this analysis. The prior distributions of historical, demographic and mutational parameters are described in Table 1. We chose to use a normal distribution for the time of ancestral divergence (based on our results from BEAST), as we were most interested in the post-divergence demographic changes for this analysis. We chose to use a generation time of four years as this falls between that of the Tasmanian devil (~3 years), and the grey wolf (*Canis lupis*, ~5 years), a species with which the thylacine shares many convergent affinities (Wroe & Milne, 2007; Wroe *et al*., 2007; Jones *et al*., 2008; Mech *et al*., 2016).

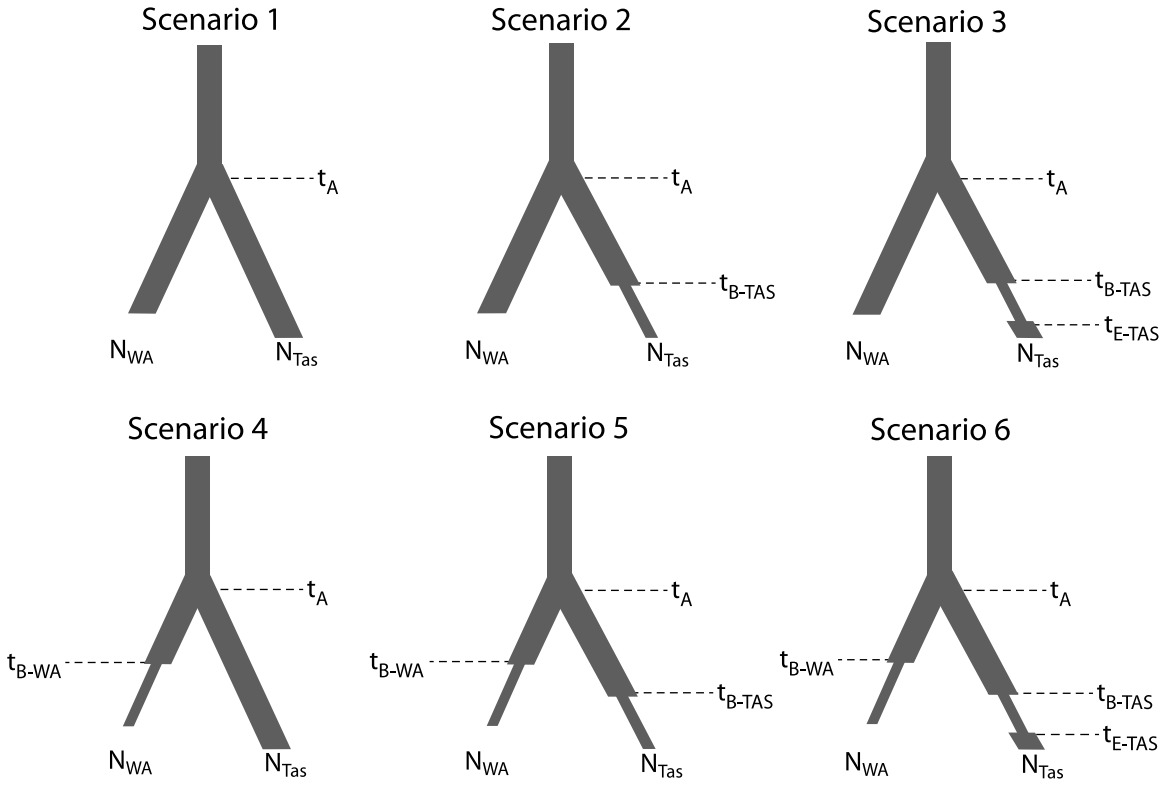

Figure 2. Demographic models tested using ABC analysis. Branch widths indicate relative population size. Dashed lines indicate time of inferred population size changes.

Table 1. Prior distributions for demographic parameters in ABC analysis. *Ne* is used for effective population size.

Interpretation	Parameter	Distribution	Min	Max	Mean	sd.	Conditions
Ne Tas (most recent)	N_{TAS}	Uniform	10	10000	-	-	-
Ne WA (most recent)	N_{WA}	Uniform	10	50000	-	-	-
Ne Tas (bottleneck)	N_{TAS1}	Uniform	10	10000	-	-	$< N_{TAS}$
Ne Tas (post divergence)	N_{TAS2}	Uniform	10	100000	-	-	$> N_{TAS1}$
Ne WA (post divergence)	N_{WA2}	Uniform	10	100000	-	-	$> N_{WA}$
Ancestral divergence time	t_A	Normal	10000	100000	30000	12000	-
Tas expansion time	$t_{E\text{-}TAS}$	Uniform	0	20000	-	-	$< t_{B\text{-}TAS}$
Tas bottleneck time	$t_{B\text{-}TAS}$	Uniform	0	40000	-	-	$< t_A$
WA bottleneck time	$t_{B\text{-}WA}$	Uniform	3200	40000	-	-	$< t_A$
Mutation model	u	Uniform	$1.00\ 10^{-9}$	$1.00\ 10^{-6}$	-	-	HKY
Mutation model	k	Uniform	0.5	20	-	-	-

Each scenario was simulated based on neutral coalescence for 10^6 iterations and summary statistics (number of haplotypes, number of segregating sites, mean and variance of pairwise differences and Fst) were computed for each simulation. DIYABC draws random values for each parameter from the prior distributions and performs coalescent-based simulations to generate simulated samples with the same number of samples and loci per population as the observed dataset. A Euclidean distance is then calculated between the summary statistics of each simulated dataset and the observed dataset (Beaumont *et al.*, 2002).

The posterior probability of each scenario was estimated using logistic regression on the 1% of simulated datasets closest to the observed dataset, subject to linear discriminant analysis as a pre-processing step (Estoup *et al*., 2012). The selected scenario was the one with the highest posterior probability value, with the 95% confidence interval (CI) not overlapping the 95% CI of any other compared scenario. We estimated the posterior distribution of each demographic parameter under the best demographic model by carrying out local linear

regression on the closest 1% of simulated data sets, after the application of logit transformation to parameter values (Cornuet *et al*., 2014).

RESULTS

Sequencing results

We successfully sequenced the mitochondrial genome (15,447 bp excluding the control region) from 51 thylacines (15 from the mainland and 36 from Tasmania, Figure 1). Thirty additional samples produced < 1000 unique reads or < 50% coverage and were excluded from further analysis. Forty-two dated samples range in age from 88 to 20,812 years BP (Table S1.1). The average coverage and depth was high for both the ancient samples (age > 600 years BP, mean coverage = 95.8%, mean depth = 152.2) and historic samples (age < 600 years BP, mean coverage = 99.5%, mean depth = 1,177.7). Full details of sequencing and mapping statistics are available in Appendix 3 (Table S3.1). All libraries showed cytosine deamination frequencies and distributions consistent with ancient or museum specimen DNA (Figure S3.1). All library and extraction blank controls had no more than two reads that mapped to the reference sequence (Table S3.1).

Descriptive Statistics and Network

Genetic diversity was lower across all measures in the historic Tasmanian thylacine population than in the ancient Tasmanian or ancient Western Australian groups (Table 2). Genetic diversity in historic Tasmanian group was also lower than in a sample of modern Tasmanian devils despite greater temporal range. The demographic estimators, Fu and Li's D* and F*, and Tajima's D, were all non-significantly negative. The shape of the pairwise

mismatch distribution suggests that the historic Tasmanian thylacine population had expanded prior to their decline to extinction (Figure 3). Using τ, we estimated the timing of this expansion to be 736 years BP.

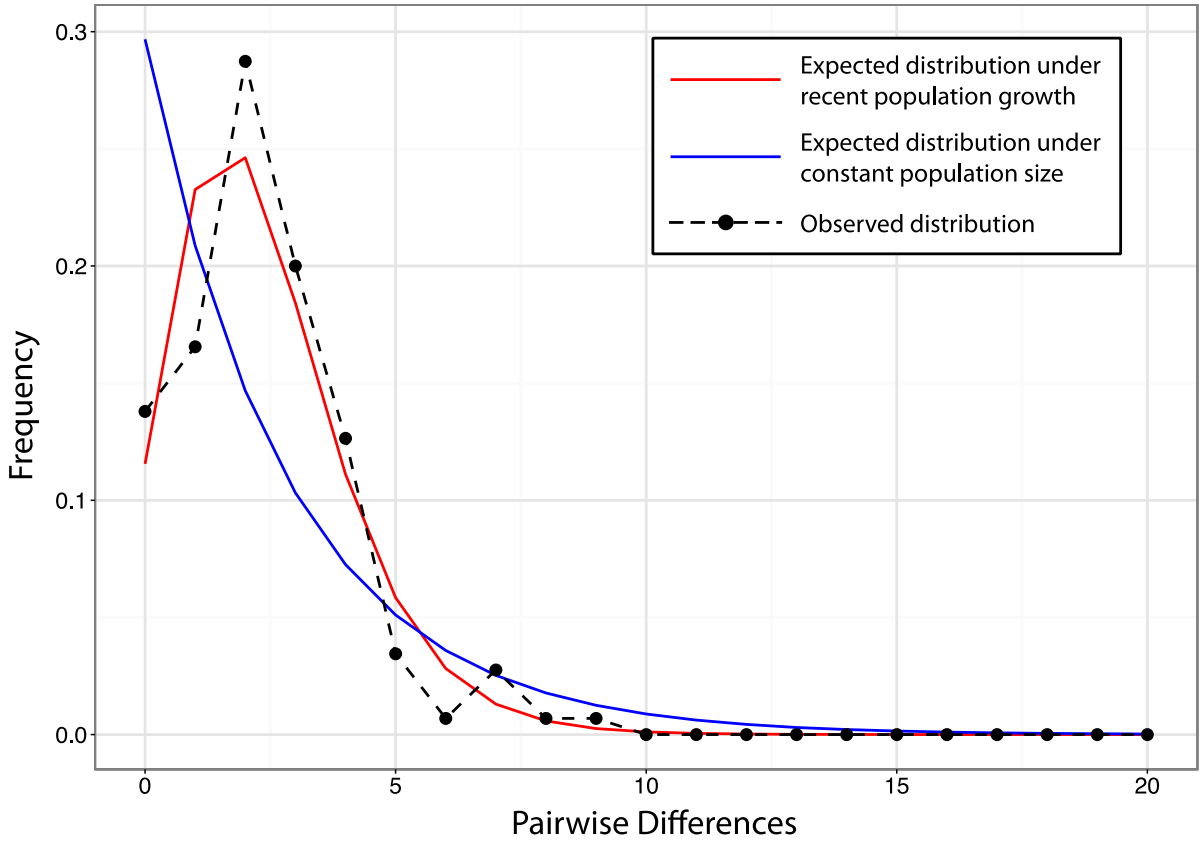

Figure 3. Mismatch distribution constructed from aligned historical Tasmanian thylacine mitochondrial sequences.

Table 2. Genetic diversity summary statistics calculated from thylacine and devil mitochondrial genomes. Number of samples (*n*), number of haplotypes (*H*), haplotype diversity (H_d), number of segregating sites (*S*), nucleotide diversity (π), average pairwise difference (*k*), Fu and Li's demographic estimators D* and F*, Tajima's D (*Td*), mode of the observed mismatch distribution (τ) and estimated time of expansion in years BP. Devil mitochondrial genomes were sourced from Miller *et al.* (2011): GenBank Accessions JX475454-67 and were modified to exclude the control region.

Group	**n**	**Age Range**	**H**	**H_d**	**S**	**π**	**k**	**D***	**F***	**Td**	**τ**	**Exp. Time**
Tas Historic	30	96-500	10	0.862	14	0.00017	2.37	-1.39	-1.52	-1.098	1.583	736
Tas Ancient	3	8-20 kya	3	1	32	0.00138	21.333	-	-	-	-	-
WA Ancient	10	3-8 kya	9	0.978	44	0.00168	14.489	-	-	-	-	-
Tas Devils	13	0-50	8	0.897	25	0.0005	7.62	0.049	0.10123	0.1961	-	-

Phylogenetic Analysis

The TCS network (Figure 4) shows two distinct groups: western thylacines versus Tasmanian and NSW thylacines. There is no structure separating the two sampling locations within the western group and the single NSW sample falls between two ancient Tasmanian samples. The undated western samples fall in with the rest of the western samples, which are genetically diverse. Three of the undated Tasmanian samples are grouped with the most frequent haplotype representing most of the historic samples. The other two undated Tasmanian samples share a haplotype with an ancient Tasmanian individual (9708) that was dated as 8,263 years old.

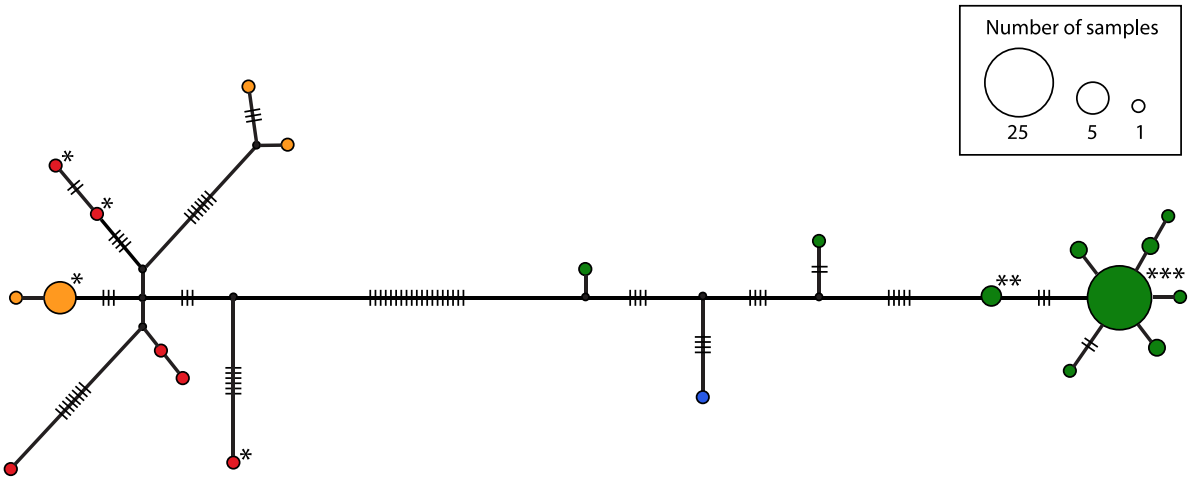

Figure 4. TCS network based on the alignment of 53 thylacine mitochondrial genome sequences (15,447 bp). Circle size is proportional to the frequency of haplotypes. Hatch marks represent the number of mutations between haplotypes. Black dots represent unsampled haplotypes and other colours relate to geographic location as presented in Figure 1 (orange= south-west WA, red=Nullarbor, WA, blue=NSW and green=Tasmania). Asterisks show the position of the nine undated ancient samples. The network was built with sites with > 5% missing data masked meaning that the number of haplotypes and mutations are underrepresented.

BEAST analyses estimated the average mutation rate to be 1.27×10^{-7} substitutions per site, per year. This rate falls within the range ($\sim 1 \times 10^{-7}$ -10^{-8}) recently estimated for numerous ancient mitochondrial DNA datasets (Ho *et al.*, 2011). The MCC tree (Figure 5) showed that the Tasmanian group (including the single NSW sample) and the western group diverged ~30,000 years BP (20,725-48,780 95% HPD). The most recent common ancestor (TMRCA) was ~12,000 years BP (8,449-16,813 95% HPD) for the western group, ~25,000 years BP (20,959-30,535 95% HPD) for the eastern group including the ancient samples, and ~1,000 years BP (455-2293 95% HPD) for the historic Tasmanian samples. The single NSW sample falls within the ancient Tasmanian samples.

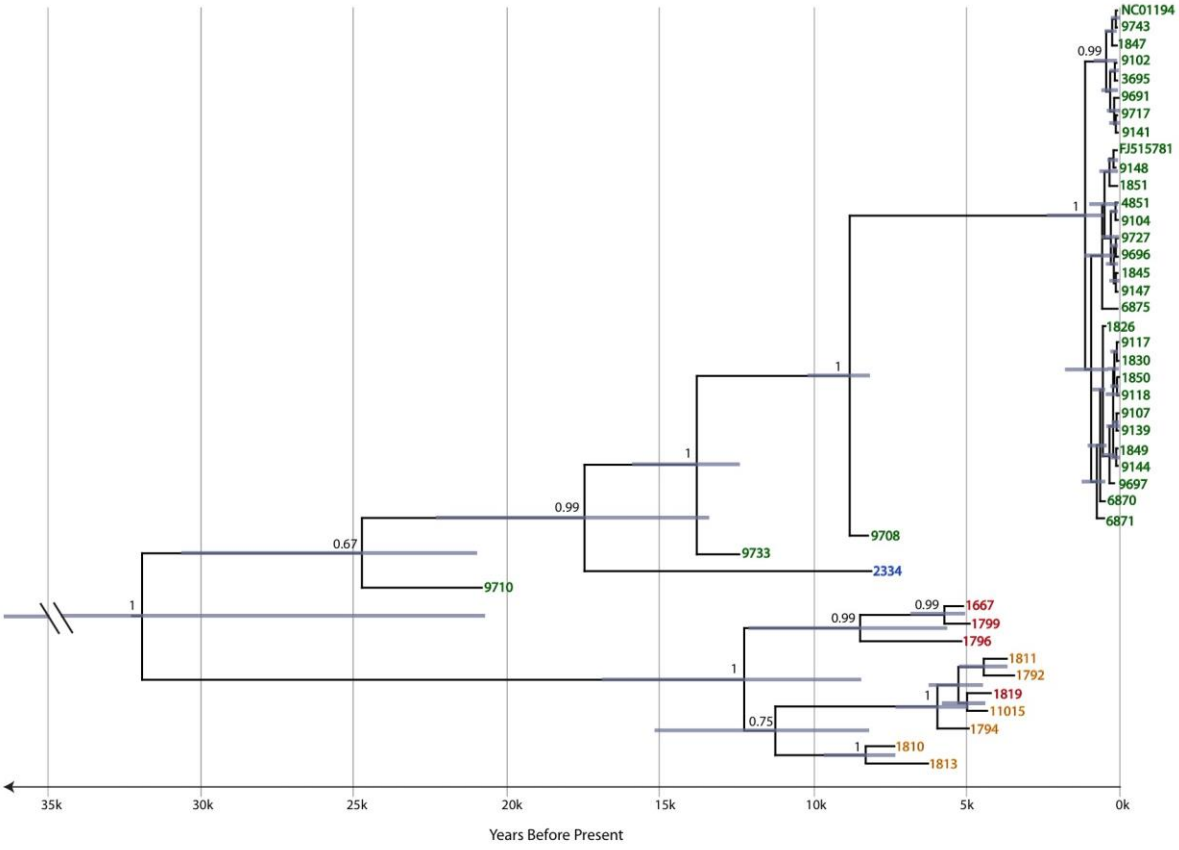

Figure 5. BEAST maximum clade credibility phylogeny of thylacine mitochondrial sequences for which radiocarbon dates were available. Nodes are labelled with Bayesian posterior probabilities (PP) for nodes with PP > 0.5. Node height reflects mean posterior age.

Grey bars at nodes represent the 95% HPD of node age. Double slanted lines indicate that a portion of the bar has been omitted because of space constraints. Colours correspond to geographic location as shown in Figure 1.

Inference of Demographic History

The coalescent-based Bayesian skyline plot shows a slow and slight decline over the last ~ 15,000 years, followed by an expansion in the Tasmanian population beginning ~1,000 years BP (Figure 6). However, confidence intervals are wide and a constant population size through time cannot be rejected.

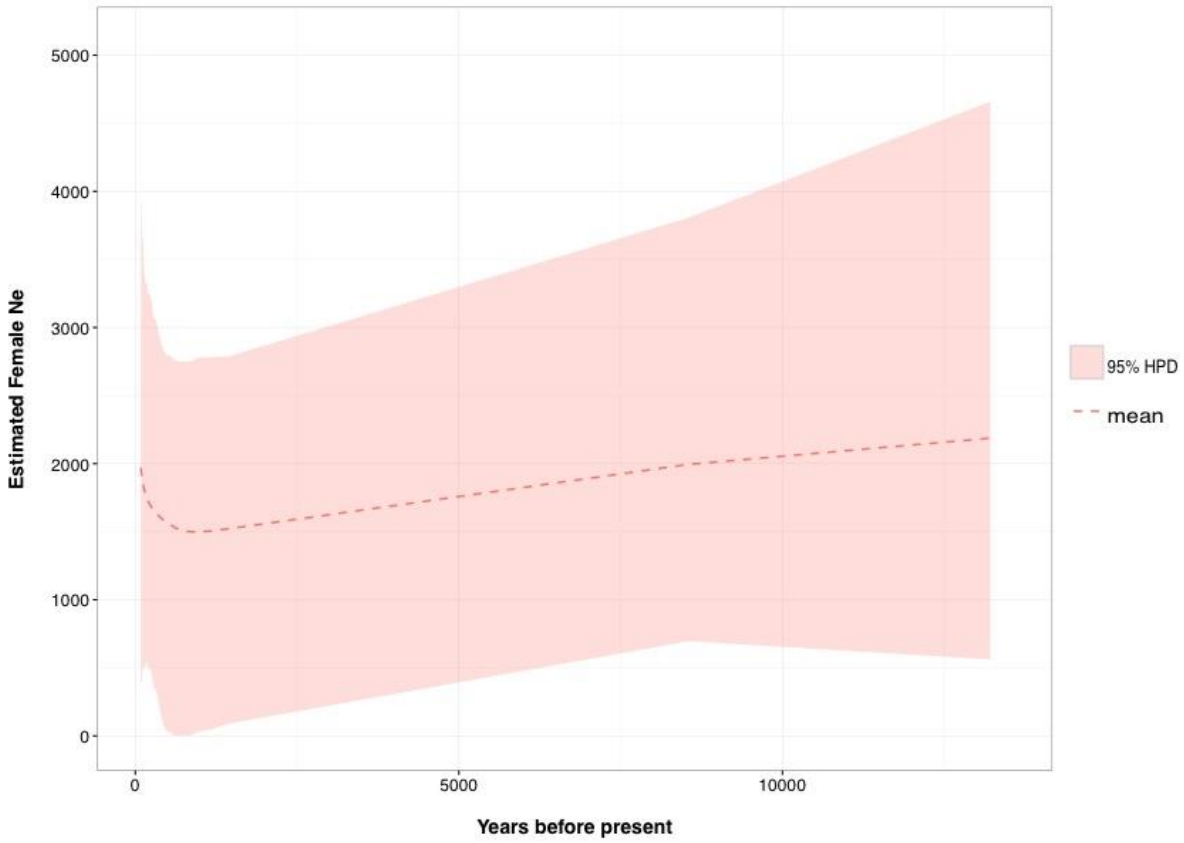

Figure 6. Extended Bayesian skyline plot of female effective population size in the Tasmanian thylacine population

ABC analysis identified Scenario 3 as the most likely scenario (Table S3.2). In Scenario 3 the population size of the western group remained constant and the Tasmanian group expanded after a bottleneck (Figure 7). The estimated parameters under Scenario 3 are given in Table 3. The timing of bottleneck and recovery in Tasmania are estimated to be 20,400 (6,440-36,520 95% CI) and 3,160 (192.8-16,960 95% CI) year BP respectively. We note that the generation time estimate used (4 years) may deviate from the thylacines true generation time, possibly biasing the timing of inferred events However, our ABC time estimates are broadly consistent with the demography inferred by our Bayesian skyline analysis.

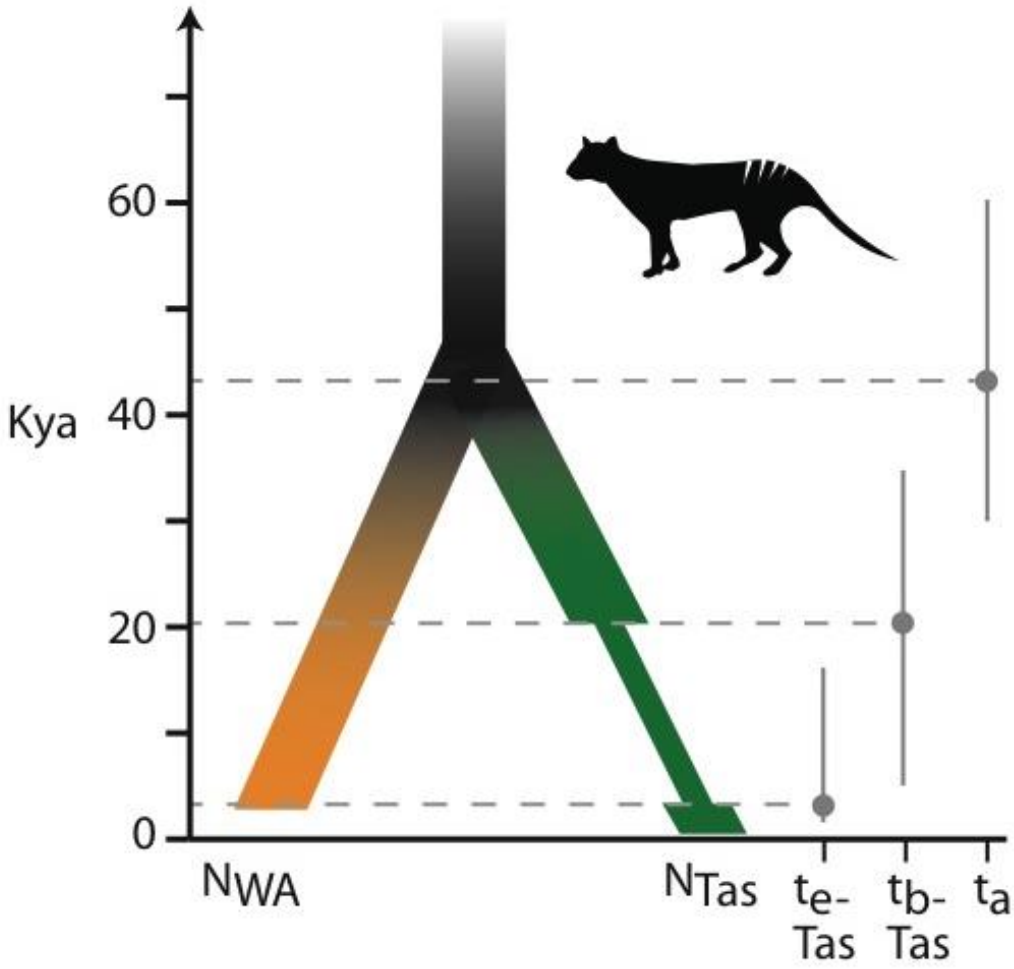

Figure 7. The thylacine demographic scenario selected by ABC analysis (Scenario 3). Time is given in thousands of years before present (Kya). Time estimates are provided as the

median and 95% confidence intervals (grey dots and error bars). The width of the branches represents relative population size.

Table 3. Posterior distributions of parameters from the selected scenario (Scenario 3).

Parameter	Median	q05	q95	Mean	Mode
N_{TAS}	**4470**	1440	9320	4850	3510
N_{WA}	**15600**	4350	42400	18500	6920
N_{TAS1}	**787**	77.2	3270	1110	67.3
N_{TAS2}	**55500**	10200	96000	54500	96300
t_A	**42400**	28160	60000	42800	39280
$t_{E\text{-}TAS}$	**3160**	192.8	16960	5400	116
$t_{B\text{-}TAS}$	**20400**	6440	36520	20880	19880
u	**$2.61\ 10^{-7}$**	$1.18\ 10^{-7}$	$5.12\ 10^{-7}$	$2.81\ 10^{-7}$	$2.61\ 10^{-7}$
k	**9.48**	0.9	18.8	9.62	1.64

DISCUSSION

Our analyses of thylacine mtDNA revealed an east-west phylogeographic split, higher genetic diversity and effective population size in western versus Tasmanian populations, and evidence for a late Pleistocene or Holocene population bottleneck and recent population expansion in the Tasmanian population.

Phylogenetic Patterns in Mainland Thylacines

The divergence between the two groups seen in our phylogenetic analysis is suggestive of isolation by distance or a demographic scenario in which the thylacines retracted into western and eastern refugia around the time of the Last Glacial Maximum (LGM, ~25,000 years BP). Evidence for retraction into east/west refugia during the LGM has been observed in a range of Australian birds (Murphy *et al.*, 2011; Dolman & Joseph, 2012) and mammals (Cooper *et*

al., 2003; Miller *et al*., 2011). The same pattern of east/west divergence has been suggested for mainland devils based on fossil occurrences, but is not observed in the fossil distribution of mainland thylacines, possibly due to taphonomic bias (Owen, 2003; Brown, 2006). The Nullarbor and/or Lake Eyre regions are well characterised biogeographic barriers for many terrestrial vertebrates and may have obstructed gene flow between populations during and after the LGM, a pattern that is evident in numerous extant vertebrate fauna (Byrne *et al.*, 2008; Austin *et al*., 2013; Marin *et al*., 2013; Neaves *et al.*, 2013). Several thylacine samples used in our study are from the Nullarbor with ages ranging from 3-7 thousand years, indicating that the western group was present on the Nullarbor immediately preceding the groups extinction. Thus, we suggest the Eyrean barrier (Lake Eyre/Flinders Ranges) as a more likely barrier for thylacines.

This apparent structuring may also be due to isolation by distance, given that eastern Australia is represented by a single mainland sample, and several mammals show evidence of Late Pleistocene range expansion across the Nullarbor and Eyrean barriers. For example, the red kangaroo, (*Macropus rufus*), western grey kangaroo (*Macropus fuliginosus*) western pygmy possum (*Cercartetus concinnus*), and fat tailed dunnart (*Sminthopsis crassicaudata*) have wide, distributions with limited genetic structure across southern Australia (Clegg *et al.*, 1998; Cooper *et al.*, 2000; Pestell *et al.*, 2007; Neaves *et al.*, 2012). Increased sampling in the east and, crucially, in southern and south-eastern Australia, will be needed to confirm whether our results show retracting populations or simply isolation by distance across the species range.

The single NSW sample falls within the eastern group, bracketed by older and younger ancient Tasmanian samples, indicating that Tasmanian and mainland populations were connected via the Bass Strait land bridge before it was flooded for a final time ~14,000 years BP (Lambeck & Chappell, 2001). The Bass Strait land bridge has acted both as a barrier and

a corridor for different terrestrial vertebrates. Koalas (*Phascolarctos cinereus*) never crossed the land bridge to reach Tasmania, whilst several mammals (e.g. Gongora *et al.*, 2012; Frankham *et al.*, 2016), frogs (e.g. Symula *et al.*, 2008) and reptiles (e.g. *Dubey & Shine, 2010)* show deep (> 0.9 MY, Pliocene/Pleistocene) divergences, suggesting ancient vicariance with no subsequent dispersal. In contrast, several other reptiles (Chapple *et al.*, 2005) and frogs (Schäuble & Moritz, 2001) crossed the land bridge in the late Pleistocene to colonise Tasmania from Victoria. More samples are needed from eastern Australia to reconstruct demographic history of thylacines in this region and to establish the extent of gene flow between Tasmania and the mainland during the late Pleistocene.

The estimated female effective population size and genetic diversity of the western population was much larger than the Tasmanian population. We do not detect any genetic patterns of decline in the Western Australian population prior to their extinction approximately 3,200 years BP. This could indicate that, like the Tasmanian thylacines, the mainland thylacine decline to extinction was rapid and not the result of intrinsic factors, such as inbreeding depression.

Tasmanian Thylacine Demographic History

The cumulative evidence from the mismatch distribution, Bayesian skyline plot, ABC analysis and the pattern of radiation of historic Tasmanian haplotypes in the BEAST tree suggest that the Tasmanian thylacine population was increasing prior to European arrival. ABC analysis suggests that this expansion represents a recovery from a population bottleneck. The 95% CI surrounding the estimated time of this bottleneck is large (6,440-36,520 years BP), possibly because ABC analysis restricts demographic scenarios to abrupt events. In contrast, the Bayesian skyline plot of the Tasmanian population suggests that the decline may have been slow and incremental, feasibly the result of the isolation of Tasmania

from the mainland. However, the CI surrounding the estimated size change is also large. While mitochondrial DNA has many properties useful for genetic analysis and can be easier to retrieve from degraded specimens, future studies should focus on multiple nuclear loci to gain more precise estimates of demographic history of the thylacines (Heled & Drummond, 2008; Ho & Gilbert, 2010).

Regardless of the mode of decline, the low genetic diversity in the Tasmanian thylacine population reveals that their effective population size was small. ABC inference suggests that the effective female population size was fewer than 1000 individuals (median = 787, 95% CI 77.2-3270) prior to the expansion, increasing to 4470 (95% CI 1440-9320) in historic times. We do not detect any genetic patterns of population decline leading up to the extinction of thylacines in 1936, likely because the extirpation occurred so quickly (Owen, 2003).

Comparison with Tasmanian Devils

The demographic history of thylacines and devils show a number of striking parallels that contrast with other terrestrial carnivores with similar distributions. Both species were widespread on the mainland during the Pleistocene but became extinct there at the same time (approximately 3,200 years BP) and both species survived a population bottleneck (or, in the thylacine's case, at least long-term low *Ne* due to island insularity), resulting in low genetic diversity in Tasmania (Brüniche-Olsen *et al*., 2014). In contrast, tiger quolls and eastern quolls (the next largest marsupial carnivores in Tasmania and eastern Australia) did not go extinct on the mainland and have higher levels of genetic diversity (Firestone *et al*., 1999). This suggests that an ecological crisis severely impacted thylacines and devils, sometime in the mid- to late-Holocene, but did not affect other marsupial carnivores. Habitat preferences (quolls favour wetter forest, while thylacines and devils were more abundant in drier, open

sclerophyll forest) may explain the contrasting response (Jones & Stoddart, 1998; Jones & Barmuta, 2000).

We cannot support or refute the hypothesis that thylacines underwent an abrupt bottleneck at the same time as devils, but we suggest that our results do support an environmental change in Tasmania at that time. The overall similarity in demographic histories suggests that a regime shift in the broad Tasmanian ecosystem caused population declines in both species. Given the absence of other drivers evident in Tasmania at the time, Brüniche-Olsen *et al*. (2014) propose the intensification of the ENSO climate system as the driver of the devils late-Holocene decline. During the late-Holocene, ENSO associated events resulted in greater variability in rainfall and increased duration and intensity of droughts across Australia (Donders *et al*., 2008). Although this climate variability is assumed to have been less pronounced in Tasmania than on the mainland (Donders *et al*., 2007), several studies of palaeoecological proxies have linked vegetation changes and fire events on the island to ENSO activity (Fletcher *et al*., 2014; Stahle *et al*., 2016; Beck *et al*., 2017).

Unstable climate, changes in vegetation states and altered fire regimes have been linked to changes in vertebrate population dynamics on the Australian mainland and other continents (Hadly, 1996; Jaksic *et al.*, 1997; Lima *et al.*, 2002; Dortch, 2004; Marshal *et al*., 2011). To test for a relationship between ENSO-linked environmental change and population size changes in the Tasmanian thylacine and devils, a greater understanding of prey abundances in Tasmania during the late-Holocene is needed.

Implications for the Devil and Thylacine Mainland Extinctions

It has been assumed that ENSO activity had minimal impact on Tasmania. However, our results and other recent studies show that climate change may have impacted the top marsupial predators on the island. Given that climate change impacts are known to have been

more severe on the mainland, this could indicate that ENSO activities have been underestimated as a potential driver of the devil and thylacine's mainland extinctions. Alternatively, the contrasting outcomes of mainland extinction and island survival may suggest that climate change alone was insufficient to cause the mainland extinctions. This is congruent with a recent simulation study that identified synergistic effects of climate change and human intensification as a probable cause of the thylacine and devil mainland extinctions (Prows*e et al.,* 2013).

Summary

Using the largest dataset of thylacine DNA sequences to date we provide the first genetic evidence that mainland thylacines split into eastern and western remnant populations in southern Australia prior to the LGM and show that the ancient western population had a larger effective population size than the recent Tasmanian population. We find no evidence for a loss of genetic diversity leading to the extinction of the western population, indicating that the mainland extinction was rapid and not the result of intrinsic factors, such as inbreeding depression.

We showed that, like devils, Tasmanian thylacines had relatively low genetic diversity, the result of a bottleneck event or island insularity. However, unlike Tasmanian devils, our analyses suggest that the Tasmanian thylacine population was expanding prior to European arrival. The timing of this expansion, in concert with a decline in Tasmanian devils and an ENSO-associated climate event, points to a possible environmental regime shift in Tasmania ~3,000 years BP. Given that ENSO effects are known to have been more severe on mainland Australia, we suggest that climate change, in synergy with other drivers (such as human intensification or dingo competition/predation), is likely to have contributed to the devil and thylacine mainland extinction.

To gain further understanding of the thylacine's demographic history and processes that led to their extinction, future studies should focus on multiple nuclear loci and strive for increased sampling in south-central and eastern mainland Australia. The Fossahul database (doi: 10.4227/05/564E6209C4FE8) of dated Australian fossils lists 32 thylacine fossils from south-central (i.e. South Australia and Victoria) and 27 from eastern (i.e. NSW and Queensland) mainland Australia (Rodríguez-Rey *et al*., 2016). While this list does not include undated material and many of the listed fossils are of an age outside the range from which it would be possible to retrieve DNA, the database shows the plausibility of filling in our sampling gaps in the future.

Climate projections predict a hotter and more arid climate across Australia in coming decades, which will exacerbate and add to existing threats to native species (CSIRO and Bureau of Meteorology, 2015). Therefore, understanding the impact of past climate change on Australian native fauna and disentangling its effects from that of human pressure and invasive species is critical for understanding extinction risk and focusing conservation efforts in the future.

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SUPPORTING INFORMATION

Appendix S1: Sample information.

Appendix S2: Expanded methods.

Appendix S3: Expanded results.

DATA ACCESSIBILITY

All DNA sequence data are deposited in GenBank accession numbers: KY678342-KY678392.

BIOSKETCH

Lauren C. White is currently a post-doctoral researcher at the Max Planck Institute for Evolutionary Anthropology. Her research there focuses on using genomic data to study kinship structure in social primate species. Her PhD thesis, completed at the University of Adelaide, focused on conservation and population genetics of Australian mammals.

Kieren J. Mitchell is interested in using ancient DNA from extinct populations and species to better understand evolutionary processes. This includes exploring how ancestral diversity is differentially inherited by daughter populations/species, inferring rates of evolution, and identifying how populations/species have reacted to past environmental change.

Jeremy J. Austin uses ancient DNA techniques through space and time to understand the evolutionary history of living and extinct mammals, reptiles and birds, to assess the impacts of past environmental change, and to provide valuable genetic data for conservation and management of threatened species.

EDITOR

Alexandre Antonelli

AUTHOR CONTRIBUTIONS

LCW acquired and analysed the data and drafted the manuscript; KJM helped analyse the data and helped revise the manuscript; JJA designed the study, collected samples, interpreted the results and helped revise the manuscript. All authors gave final approval for publication

Journal of Biogeography

SUPPORTING INFORMATION

Ancient mitochondrial genomes reveal the demographic history and phylogeography of the extinct, enigmatic thylacine (*Thylacinus cynocephalus*)

Lauren C. White, Kieren J. Mitchell, and Jeremy J. Austin

APPENDIX S1: Sample details

Table S1.1 Origin, museum ID and age details of thylacine samples used in this study. Calibrated ages are given for the 19 subfossil samples that were radiocarbon dated, the age of museum samples was calculated from the collection date where available. Genetic, geographic and date details confirm that all listed samples come from different individuals.

Sample Number	State	Location	Sample Type	Museum	Museum ID	Radiocarbon ID	Cal. Age: Years BP	SD of Cal. Age	Age Years BP (Museum Samples)	Lat.	Long.
1667	WA	Nail Tail Cave, Rawlinna Station	bone	Western Australian Museum	04.2.359	ANU-43714	5163	82	-	-31.005	125.52
1792	WA	Foundation Cave, Augusta	bone	Western Australian Museum	04.2.280	ANU-47315	3498	44	-	-34.322	115.14
1794	WA	Foundation Cave, Augusta	bone	Western Australian Museum	04.2.249	ANU-43716	4976	87	-	-34.322	115.14
1796	WA	Nail Tail Cave, Rawlinna Station	tooth	Western Australian Museum	04.2.347	ANU-43717	5167	81	-	-31.005	125.52
1797	WA	Nail Tail Cave, Rawlinna Station	tooth	Western Australian Museum	04.2.348	-	-	-	-	-31.005	125.52
1799	WA	Weebubbie Cave	bone	Western Australian Museum	04.2.463	ANU-43719	4976	87	-	-31.662	128.78
1804	WA	Nail Tail Cave, Rawlinna Station	bone	Western Australian Museum	04.2.358	-	-	-	-	-31.005	125.58
1810	WA	Skull Cave	tooth	Western Australian Museum	69.9.11	ANU-43730	7407	49	-	-34.200	115.08
1811	WA	unnamed cave, c. 400m south of Deepdene Cave	tooth	Western Australian Museum	73.7.132	ANU-43732	3737	59	-	-33.97	115.02
1813	WA	Skull Cave	tooth	Western Australian Museum	69.12.3	ANU-43729	6333	41	-	-34.200	115.08
1819	WA	Murra-el-elevyn Cave	skin	Western Australian Museum	64.8.1	ANU-43739	4261	76	-	-31.703	127.73
1826	TAS	Croesus Cave	bone	Queen Victoria Museum and Art Gallery	1993.GFV.8	ANU-43725	530	13	-	-41.573	146.22
1830	TAS	-	tooth	South Australian Museum	M922	-	-	-	-	-	-

1845	TAS	-	tooth	Museum Victoria	C5741	-	-	-	-	-	-
1847	TAS	-	tooth	Museum Victoria	C5745	-	-	-	-	-	-
1849	TAS	-	tooth	Museum Victoria	C5742	-	-	-	-	-	-
1850	TAS	Arthurs Lakes, Western Tiers	tooth	Museum Victoria	C5746	-	-	-	-	-41.98	146.92
1851	TAS	northern Tasmania	bone	Museum Victoria	C5750	-	-	-	-	-	-
2334	NSW	Janus Cave	tooth	ANWC	CM15266	WK-36184	8108	48	-	-35.716	148.5
3695	TAS	Central Highlands	skin	Tasmanian Museum and Art Gallery	A1298	-	-	-	-	-42.67	146.47
4851	TAS	-	bone	South Australian Museum	M95	ANU-43726	117	84	-	-	-
6870	TAS	Mole Creek Caves	bone	Queen Victoria Museum and Art Gallery	QVM:1992:GFV:222	ANU-50329	531	11	-	-41.574	146.29
6871	TAS	Mole Creek Caves	bone	Queen Victoria Museum and Art Gallery	QVM:1992:GFV:1	ANU-43727	543	21	-	-41.266	146.81
6875	TAS	Flowery Gully, Tamar Valley	bone	Queen Victoria Museum and Art Gallery	QVM:1976:1:86	-	-	-	-	-41.266	146.81
9102	TAS	-	tooth	American Museum of Natural History	AMNH 144316	-	-	-	150	-	-
9104	TAS	-	tooth	American Museum of Natural History	AMNH 173500	-	-	-	-	-	-
9107	TAS	-	tooth	American Museum of Natural History	AMNH 35504	-	-	-	-	-	-
9117	TAS	-	toepad	Oxford University Museum of Natural History	OUM 4794	-	-	-	-	-	-
9118	TAS	-	toepad	Oxford University Museum of Natural History	OUM 7934	-	-	-	-	-	-
9139	TAS	-	skin	Leeds Museum	LeedM.C.1869.46.7	-	-	-	-	-	-
9141	TAS	-	tooth	Leeds Museum	LeedM.C.1869.46.5.4091	-	-	-	-	-	-
9144	TAS	-	tooth	Leeds Museum	LeedM.C.1869.46.4.4087	-	-	-	-	-	-
9147	TAS	-	toepad	Swedish Museum of Natural History	NRM 590213	-	-	-	125	-	-

9148	TAS	-	tissue	Swedish Museum of Natural History	NRM 566599	-	-	-	173	-	-
9688	TAS	St Helens Point	bone	Tasmanian Museum and Art Gallery	A1317	-	-	-	-	-41.28	148.36
9691	TAS	northwest coast	tooth	Tasmanian Museum and Art Gallery	A295	-	-	-	96	-	-
9696	TAS	northwest coast	tooth	Tasmanian Museum and Art Gallery	A300	-	-	-	96	-	-
9697	TAS	Zulu Cave, Florentine River	tooth	Tasmanian Museum and Art Gallery	A890	ANU-50333	201	46	-	-42.63	146.44
9700	TAS	northwest coast	tooth	Tasmanian Museum and Art Gallery	A297	-	-	-	-	-	-
9706	TAS	March Fly Pot cave, Huon Valley	bone	Tasmanian Museum and Art Gallery	A2524	-	-	-	-	-43.4	146.9
9707	TAS	March Fly Pot cave, Huon Valley	bone	Tasmanian Museum and Art Gallery	A2527	-	-	-	-	-43.4	146.9
9708	TAS	March Fly Pot cave, Huon Valley	tooth	Tasmanian Museum and Art Gallery	A2525	ANU-50330	8263	48	-	-43.4	146.9
9709	TAS	March Fly Pot cave, Huon Valley	bone	Tasmanian Museum and Art Gallery	A2526	-	-	-	-	-43.4	146.9
9710	TAS	March Fly Pot cave, Huon Valley	tooth	Tasmanian Museum and Art Gallery	A2316	ANU-50331	20812	136		-43.4	146.9
9717	TAS	Warratah, NW Tas	toepad	Tasmanian Museum and Art Gallery	A1299	-	-	-	88	-41.45	145.53
9733	TAS	Florentine Valley, Derwent Valley	bone	Tasmanian Museum and Art Gallery	A1401	ANU-50332	12501	74	-	-42.73	146.67
9727	TAS	Tyenna, Derwent Valley	bone	Tasmanian Museum and Art Gallery	A315	-	-	-	-	-42.67	146.47
9743	TAS	Ouse, Central Highlands	bone	Tasmanian Museum and Art Gallery	A321	-	-	-	142	-42.49	146.71
11015	WA	Skull Cave	bone	Western Australian Museum	69.12.13	ANU-43728	4436	63	-	-34.200	115.08
11028	WA	Thylacine Hole, Mundrabilla Station	bone	Western Australian Museum	F6353	-	-	-	-	-31.703	127.73
11038	WA	Jewel Cave	bone	Mike Bunce (Curtin University)	MB127/AD 167	-	-	-	-	-34.274	115.09

APPENDIX S2: Expanded methods

DNA Extraction

We performed DNA extraction as per Brotherton *et al*., (2013), with some small changes: bone and tooth samples were powdered using a Mikro-dismembrator (Sartorius: Goettingen, Germany) in sterilised stainless steel containers. For each sample, 0.5-0.25 g of bone/tooth powder or 1-2 cm^2 of dried tissue was digested overnight, under constant rotation at 55 °C. Bone/tooth powder was digested in 4 mL of 0.5 M EDTA (pH 8.0), 0.5% SDS and 0.2 mg/ml proteinase K. Tissue samples were digested in 2 mL of digestion buffer containing 1.53 mL H20, 20 mM NaCl, 75 mM Tris (pH 8.0), 0.75% SDS, 50 mM DTT and 0.5 mg/ml proteinase K (all Sigma–Aldrich: St-Louis, MO, USA).

After lysis, samples were centrifuged at 4,600 rpm for 2 minutes and the supernatant transferred to a 50 mL tube containing 100 μL of medium–sized silica suspension and 16 mL (bone/tooth samples) or 8 mL (tissue samples) of modified binding buffer containing 13.5 mL QG buffer (Qiagen: Venlo, Netherlands), 1.3% Triton X–100, 25 mM NaCl, 170 mM ammonium acetate (all Sigma–Aldrich). DNA was left to bind to the silica at room temperature under constant rotation. After one hour, the silica particles were pelleted by centrifuging the mix at 4500 rpm for 2 minutes and the supernatant was discarded. The silica was then transferred to a 2 mL tube and washed twice in 1 mL of 80% ethanol, centrifuging at 14,000 rpm between washes. The pellet was air-dried on a heat block at 37 °C for 15 minutes and the DNA eluted twice with 50 μL EB buffer (Qiagen) with 0.05% Tween 20 (Sigma-Aldrich), pre-warmed to 50 °C. After pelleting for 1 minute at 14,000 rpm the supernatant was collected, aliquoted and stored at -20 °C until further use. An extraction blank control was included in each batch of extracts

Hybridisation Enrichment

One round of hybridisation enrichment was performed on each library prepared extract using thylacine mitochondrial genome RNA baits commercially synthesized by MYcroarray (MI, USA) following the manufacturers protocols with some modifications: We used P5/P7 blocking RNA oligonucleotides (Table S2.1) instead of blocking nucleotides provided with the kit. Libraries made from ancient samples were enriched using one quarter the amount of RNA baits suggested in the manual, allowing each library to be enriched separately to minimise competition for baits and bias in subsequent PCR amplifications. Hybridisation of the ancient libraries was conducted at 55 °C for 25 hours to increase the capture of short fragments. Historic sample and blank control libraries were pooled at equal concentrations into groups of three to seven samples prior to enrichment and hybridisation was conducted at 65 °C for 25 hours. Finally, we incubated the magnetic beads with yeast tRNA to saturate all potential non-specific sites on the magnetic beads that could bind nucleic acids and therefore decrease final DNA yield.

We used a short-cycle PCR additional to the manufacturers protocol to increase total DNA yield after enrichment for all libraries, except for 18 historic sample libraries that had high DNA quantity prior to enrichment. Post enrichment PCR amplifications were performed in five separate reactions containing 3 μL of captured library, 1x Kapa Hifi Hot Start Ready Mix (Kapa Biosystems: Boston, MA, USA), 300 μM of each primer (Table S2.1), and water to 25 μL. Thermocycling consisted of 98 °C for 30 seconds followed by 7 cycles of 98 °C for 20 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and a final extension of 72 °C for 5 minutes. The PCR products were pooled and DNA was purified using AMpure magnetic beads (Agencourt®, Beckman Coulter: Fullerton, CA, USA) or a homemade mix created by combining Carboxyl-modified Sera-Mag Magnetic Speed-beads (Fisher Scientific: Pittsburgh, PA, USA) in a PEG/NaCl buffer (Rohland and Reich, 2012).

The addition of full-length Illumina sequencing adapters was performed in five separate PCR reactions each as above, but using the products from the first post-enrichment PCR (or directly from the captured product in the case of the 18 historic samples) with primers matching the truncated adapter sequences (Table S2.1). The PCR products were again pooled and DNA was purified as above.

Table S2.1 Primer sequences used in this study. * indicates primers taken from Meyer and Kircher, (2010)

Primer	**Primer Sequence (5'-3')**
GAII_Indexing_X*	CAAGCAGAAGACGGCATACGAGATNNNNNNNGAGTGACTGGAGTTCAGACGTGT
IS4_indPCR_P5*	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
IS7_short_amp_P5*	ACACTCTTTCCCTACACGAC
IS8_short_amp_P7*	GTGACTGGAGTTCAGACGTGT
P5_short_RNAblock	ACACUCUUUCCCUACACGAC
P7_short_RNAblock	GUGACUGGAGUUCAGACGUGU

Table S2.2 AICM scores comparing the extended Bayesian skyline (ESB) and constant population (CP) tree priors. The lowest score (and therefore the favoured model) is in bold.

	AICM	**S.E. (+/-)**	**ΔAICM**
EBS	**43096.684**	0.045	0
CP	43098.547	0.051	1.863

Table S2.3 The optimal partitioning scheme identified by PARTITIONFINDER 1.1.1 and used in the BEAST 2.4.1 analysis

Partition	**Model**	**Positions**
1	HKY	Codon position one of ATP6, ATP8, ND1, ND2, ND4L, ND4, ND5 and ND6. Codon position 2 of ND6. Plus 12srRNA, 16srRNA and trRNA
2	F81	Codon position two of ATP6, ATP8, COX1, COX2, COX3, CytB, ND1, ND2, ND3, ND4L, ND4 and ND5.
3	TrN	Codon position three of ATP6, ATP8, COX1, COX2, COX3, CytB, ND1, ND2, ND33, ND4L, ND4, ND5 and ND6. Plus the non-coding regions.
4	K80	Codon position one of COX1, COX2, COX3, CytB and ND3.

Figure S2.1. Results of the date randomization test. The red circle and lines represent the mean and 95% HPD estimates of the average molecular rate obtained in the phylogenetic analysis of thylacine mitogenomes. The black circles and lines represent the mean and 95% HPD intervals of average rates estimated with randomized dates. None of the margins overlap with the rate estimate from the original data set, demonstrating that the radiocarbon dates used for this study contain sufficient temporal information for calibrating the molecular clock.

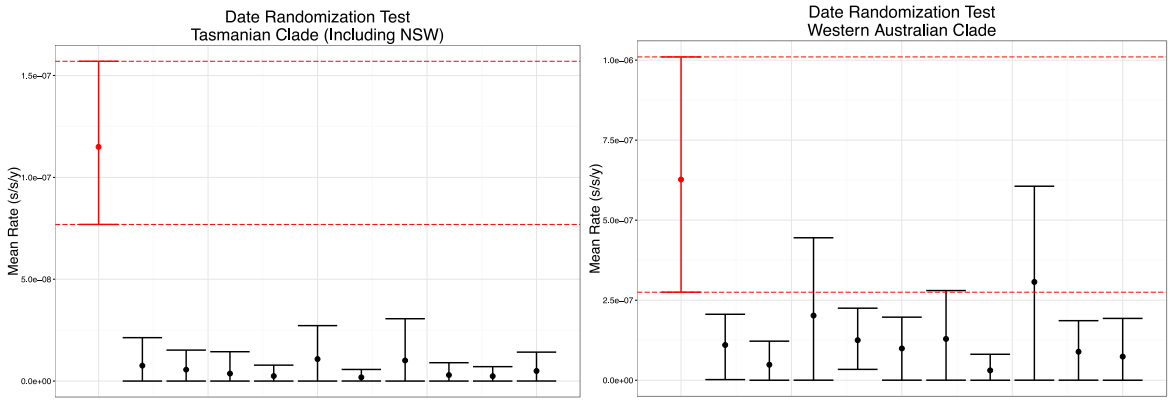

Figure S2.2. Date randomization test of the two identified clades separately. The red circle and lines represent the mean and 95% HPD estimates of the average molecular rate obtained in the phylogenetic analysis of thylacine mitogenomes. The black circles and lines represent the mean and 95% HPD intervals of average rates estimated with randomized dates. The Tasmanian/NSW group contain sufficient temporal information to be run alone, but the Western Australian group does not.

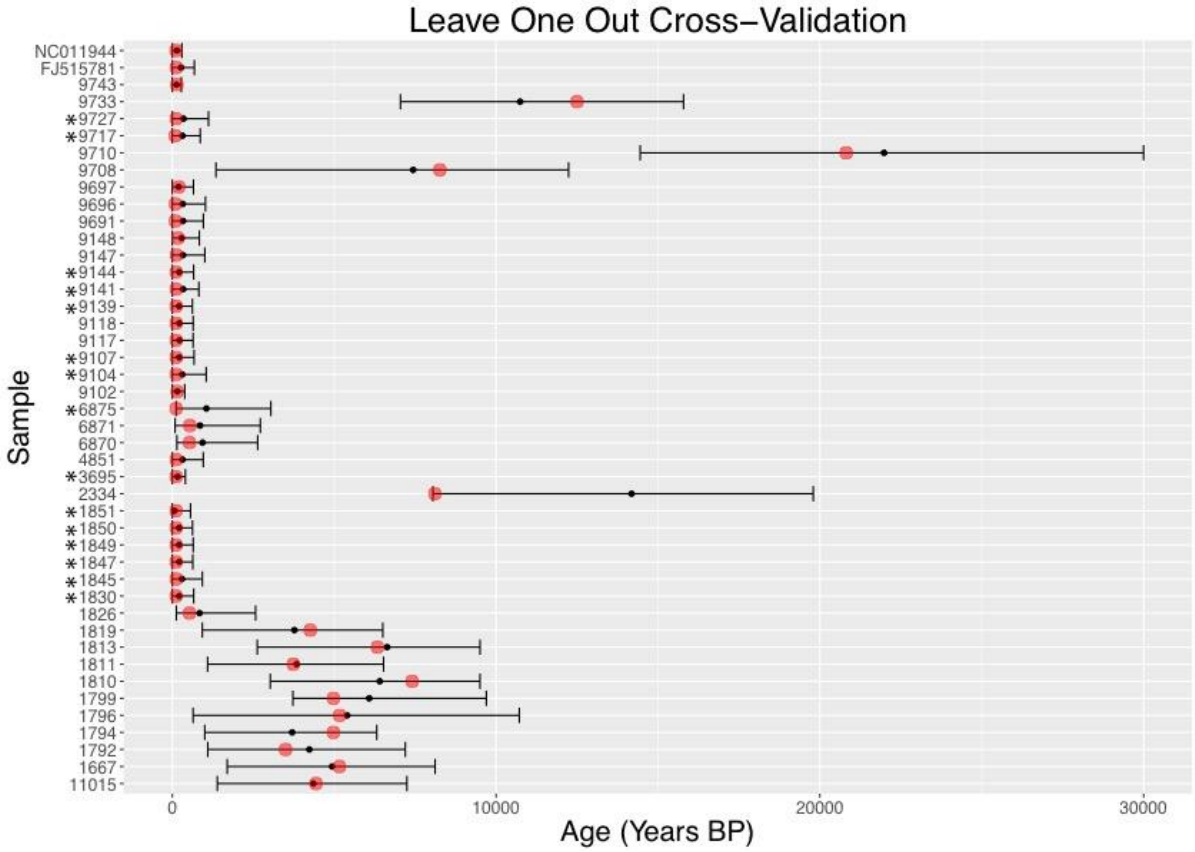

Figure S2.3 Leave one out cross-validation test. Black dot and bars represent the median and 95% HDP of the estimated age for each sample. Red dots represent the mean calibrated radiocarbon age of ancient samples, and known or estimated collection date for historic museum samples used in our BEAST analysis. All ages used in the BEAST analysis are contained within the 95% HDP estimated by LOOCV, meaning that the test did not detect any bias stemming from sequencing error, post-mortem DNA damage, or dating error. Asterisks mark historic samples for which we estimated collection dates.

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APPENDIX S3: Expanded Results

Table S3.1. Sequencing and mapping summaries of sequenced samples, extraction blank controls (EBC) and library blank controls (LBC) mapped against NC011944 excluding the control region

Sample	Raw Reads	Mapped Reads	Unique Mapped Reads	Coverage	Coverage (sites with >3 reads)	Average Depth
1667	5174560	2146375	55289	100	100	212.3
1792	1553643	366578	1102	95.9	80.4	5.6
1794	3422828	1529283	8892	100	100	47
1796	5859883	784015	3222	91.7	73.9	10.2
1797	1089746	582238	13516	100	99.9	40
1799	1214844	298231	73599	100	100	341
1804	800089	181013	3312	98.3	88.7	9.8
1810	5066187	447815	3440	100	99.8	15.3
1811	8044948	2509990	29917	100	100	141.9
1813	1616715	120837	1335	99.3	87.8	5.6
1819	3255801	561342	35658	100	100	108.7
1826	1124170	821945	27302	100	100	140.3
1830	16251960	12076112	26658	100	100	156.8
1845	11151324	6757820	91642	100	100	663.1
1847	30050688	19617087	791788	100	100	6143
1849	1916154	1069065	4076	100	100	34.2
1850	1246318	559748	344491	100	100	2806.5
1851	21717751	17676404	2329	98.3	95.7	17.4
2334	3782410	943826	23262	100	100	131.3
3695	4032587	274837	1640	99.9	94.8	9.1
4851	3457007	1477114	520474	100	100	3579.2
6870	3381745	141688	5812	100	100	32.6
6871	2309514	1205700	14382	100	100	87.3
6875	12231456	2760521	2425	100	98.4	15.6
9102	5035667	3655873	28552	100	100	158.1
9104	3554763	1493213	36417	100	100	237.2
9107	3281279	2306987	7455	100	100	51.2
9117	1447123	414469	288483	100	100	1446.6
9118	1851219	644012	464358	100	100	2653.6
9139	360929	90592	54408	100	99.5	139.8
9141	3845624	2822344	89263	100	100	641.7
9144	10827724	8427329	132651	100	100	1067
9147	849166	292803	234946	100	100	1303
9148	772220	294354	220742	100	100	1179.4
9688	38339	3489	2387	100	97.9	12.7
9691	898370	319415	228096	100	100	1637.7

9696	720837	192730	168550	100	100	1470.6
9697	843780	378166	287615	100	100	2195
9700	102141	4879	1653	99.4	89.1	6.4
9706	221529	91669	43367	100	100	308.8
9707	73347	12603	1407	100	97.2	8.4
9708	634149	307589	237520	100	100	1520.2
9709	71706	1848	1154	99	90.7	6.5
9710	97257	25137	21375	100	100	93.2
9717	659670	214822	180944	100	100	987.6
9733	1891433	904445	40278	100	100	251.9
9727	162040	46861	254056	100	100	1871
9743	2291227	1115999	594665	100	100	3417
11015	18560536	12294077	5843	100	100	26.8
11028	2418082	1042954	12290	100	100	39.8
11038	1001149	284646	3854	100	99.8	16.7
EBC_1	47394	1	1	-	-	-
EBC_2	55792	7	2	-	-	-
EBC_3	782	0	0	-	-	-
EBC_4	113898	0	0	-	-	-
EBC_5	2975	5	1	-	-	-
LBC_1	124358	0	0	-	-	-
LBC_2	9538	0	0	-	-	-
LBC_3	21711	0	0	-	-	-
LBC_4	1481	0	0	-	-	-
LBC_5	114277	0	0	-	-	-
LBC_6	91254	0	0	-	-	-
LBC_7	85197	0	0	-	-	-
LBC_8	340953	0	0	-	-	-

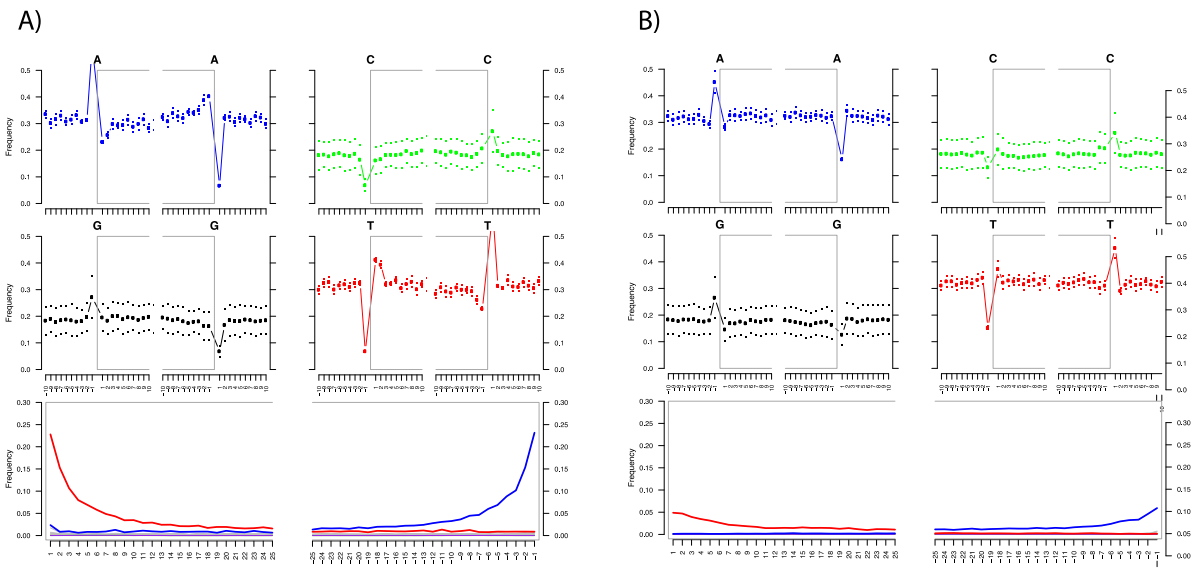

Figure S3.1. Example MAPDAMAGE2 plots. The top four plot show the characteristic high frequency of purines immediately prior to the strand break. The bottom two panels show the characteristic accumulation of 5' C-to-T (red) and 3' G-to-A (blue) misincorporations. A) An example of an ancient sample (sample 1799) and B) an example of a historic museum sample (sample 1850).

Table S3.2. DIYABC comparison of scenarios. The selected scenario was the one with the highest posterior probability with a 95% CI that does not overlap any other scenario (highlighted in bold).

Scenario	Probability Values [95% Confidence Intervals]
Scenario1	0.1848 [0.1711,0.1985]
Scenario2	0.1664 [0.1564,0.1764]
Scenario3	**0.2748 [0.2640.0.2856]**
Scenario4	0.1128 [0.1047,0.1208]
Scenario5	0.0953 [0.0866,0.1039]
Scenario6	0.1660 [0.1604,0.1716]

Chapter 5

Evaluating the genetic consequences of reintroduction in four threatened mammal species at Arid Recovery Reserve

Manuscript prepared for submission

Statement of Authorship

Title of Paper	Evaluating the genetic consequences of reintroduction in four threatened mammal species at Arid Recovery Reserve
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript prepared in publication format

Principal Author

Name of Principal Author (Candidate)	Lauren C White
Contribution to the Paper	Generated and analysed the data, helped interpret the results, drafted the manuscript and produced the figures.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/3/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Katherine E. Moseby
Contribution to the Paper	Helped design the study, collect samples, interpret the results and revise the manuscript.
Signature	Date 30th March 2017

Name of Co-Author	Vicki A. Thomson
Contribution to the Paper	Helped generate and analyse the data, interpret the results and revise the manuscript.
Signature	Date 31/3/17

Name of Co-Author	Steve Donnellan
Contribution to the Paper	Provided samples stored at the South Australian museum, helped with analysis, helped interpret the results and helped revise the manuscript.
Signature	Date 31/3/17.

Name of Co-Author	Jeremy J. Austin
Contribution to the Paper	Helped design the study, interpret the results and revise the manuscript.
Signature	Date 31/3/17

Evaluating the genetic consequences of reintroduction in four threatened mammal species at Arid Recovery Reserve

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Abstract

Reintroduction programs aim to restore self-sustaining populations of threatened species to their historic range. However demographic restoration may not reflect genetic restoration, which is necessary for the long-term persistence of populations. The Arid Recovery Reserve is a reintroduction site where four threatened Australian mammals, the greater stick-nest rat (*Leporillus conditor*), greater bilby (*Macrotis lagotis*), burrowing bettong (*Bettongia lesueur*) and western barred bandicoot (*Perameles bougainville*), have been reintroduced. These reintroductions at Arid Recovery have been deemed successful based on the growth and persistence of the populations, however the genetic consequences of the reintroductions are not known. Eighteen years after the first reintroductions to the reserve, we have generated large, high-resolution single nucleotide polymorphism (SNP) datasets for each reintroduced population at Arid Recovery today and compared them to samples taken from their founders at the time of release. We found that average genetic diversity in all populations at the Arid Recovery Reserve are close to, or exceeding, the levels measured in their founding groups. Increased genetic diversity was achieved by admixing slightly diverged and inbred source populations. Our results suggest that genetic diversity in translocated populations can be improved or maintained over relatively long time frames, even in small fenced conservation reserves and highlight the power of admixture as a tool for conservation management to maximise genetic diversity in threatened taxa.

Introduction

Reintroduction programs aim to establish self-sustaining populations that do not require significant long-term management, and can be an effective tool in the conservation of threatened wildlife (IUCN, 2016). Successful reintroductions generally increase the population size and geographic range of a species, and restore ecological function to the area from which it had been extirpated (Armstrong *et al*., 2015). Measuring an increase in population growth and size is most often how these reintroduction programs are judged to have succeeded (Ewen *et al*., 2012; Moseby *et al*. 2011). However, the ability of a population to persist in the long-term will also be strongly influenced by levels of genetic diversity (Cochran-Biederman *et al*., 2014, Weeks *et al*., 2015).

Reintroduced populations are susceptible to loss of genetic diversity due to founder effects, the isolated nature of reintroduction sites, and small population size (Frankham *et al*., 2010). These circumstances result in unavoidable inbreeding and stochastic loss of alleles (genetic drift). Loss of genetic diversity can then lead to reduced fitness through the accumulation of deleterious alleles (genetic load), and the increased expression of recessive deleterious traits (inbreeding depression). Additionally, the loss of genetic diversity will diminish the adaptive capacity of a population and limit its ability to cope with environmental change (Groombridge *et al.*, 2012).

Thus, most reintroduction programs adopt the preservation of genetic diversity as an explicit goal. Several best practice guidelines can be followed to maximise genetic diversity in reintroduced populations, such as using large numbers of genetically diverse individuals in the first stage of a reintroduction (founders) and encouraging rapid population growth after establishment (Jamieson and Lacy, 2012). However, it may not always be possible to follow these guidelines and many other interacting factors, such as the life-history traits and

demographic history of a species, may affect genetic diversity in cryptic ways. It is therefore important that genetic monitoring is used in all reintroduction programs to evaluate success and guide management actions that will maximise the retention of genetic diversity (Schwartz *et al.*, 2007).

Most published studies assessing change in genetic diversity in reintroduction programs have done so by sampling the source and reintroduced populations simultaneously a number of years after release—for example *Gongylomorphus bojerii*. (Michaelides *et al.,* 2015) and *Notionmystis cincta* (Brekke *et al*., 2011)—or by sampling just the reintroduced population at multiple time-points—such as *Vulpes velox* (Cullingham and Moehrenschlager, 2013) and *Mustela nigripes* (Cain *et al.,* 2011). Despite the critical importance of genetic monitoring in reintroduction programs, relatively few studies have explicitly tested changes in genetic diversity from founders to descendants over multiple generations (*e.g.* Maraes *et al.,* 2017). Such data is crucial for validating and establishing guidelines for maximising genetic diversity in reintroduced populations.

The Arid Recovery Reserve reintroduction program provides a model system in which to compare founder and descendent genetic diversity, as tissue samples were taken from founding individuals at time of release and stored explicitly for later genetic analysis. The reserve is a 123 km^2 fenced exclosure situated 20 km north of Roxby Downs in arid South Australia (Figure 1). A netting fence surrounds the reserve, and all European rabbits (*Oryctolagus cuniculus*), cats (*Felis catus*), and foxes (*Vulpes vulpes*) have been removed from a 60 km^2 sector at the southern end (Moseby and Read, 2006). Since 1998, this has allowed four species of locally extinct mammals to be reintroduced within the exclosure (Moseby *et al.* 2011), namely the greater stick-nest rat (GSNR, *Leporillus conditor*), greater bilby (*Macrotis lagotis*), burrowing bettong (*Bettongia lesueur*), and western barred bandicoot (WBB, *Perameles bougainville*). These species were all once widespread across

the Australian arid zone, but their geographic ranges have been severely reduced due to competition with grazing stock and rabbits, and predation from introduced cats and foxes (Burbidge and McKenzie, 1989; Morton, 1990; Newsome, 1971).

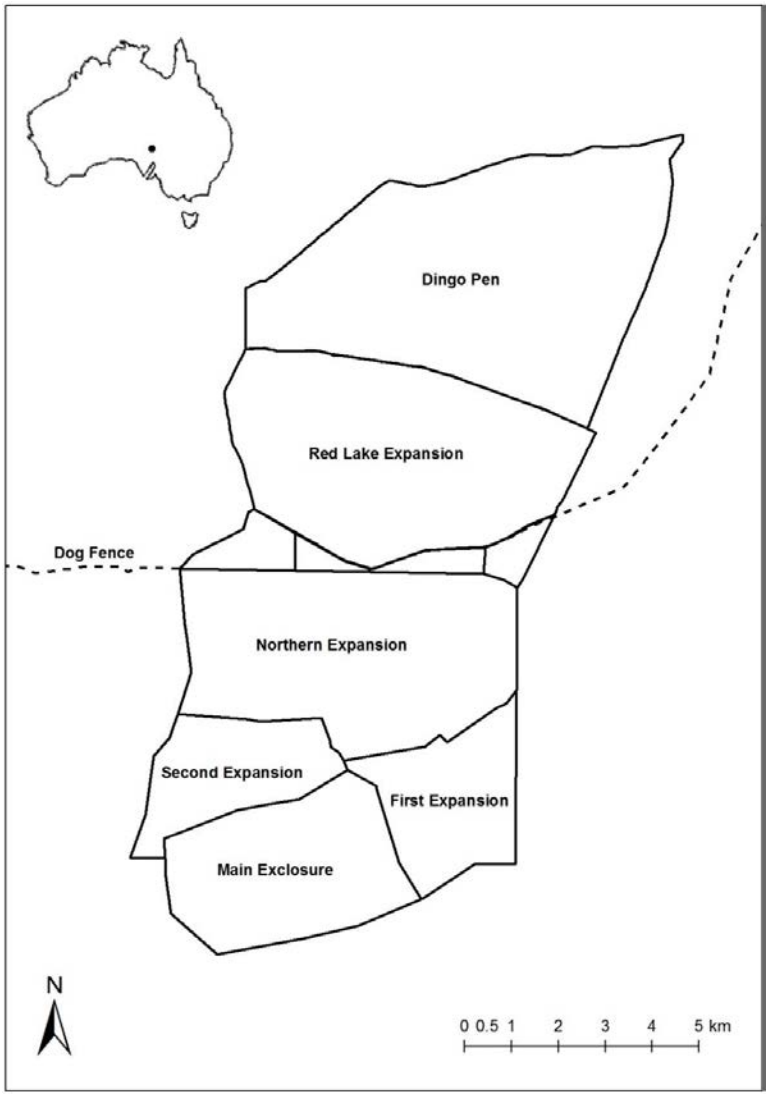

Figure 1. Location and lay-out of Arid Recovery reserve. Rabbits, cats and foxes have been removed from the four southern paddocks of the Reserve

The reintroductions at Arid Recovery have been deemed successful based on short and medium-term criteria, such as continued survival, population recovery after drought and increased abundance and distribution within the reserve (Moseby *et al.*, 2011). Annual

population estimates have shown that all four species' populations have expanded since release, although the GSNR and bilby populations have fluctuated significantly. However, the small number of animals used to found the Arid Recovery populations (n=17-122) and fluctuating population growth patterns in some species make loss of genetic diversity and inbreeding depression a concern, raising practical questions about the need for additional translocations (i.e. genetic rescue).

Here we measure the change in genetic diversity between founders and the descendant populations 18 years after the first reintroductions at Arid Recovery and 7 years since the last animal was released. We generated large single nucleotide polymorphism (SNP) datasets for samples from the founding individuals (where available) and from the contemporary, descendant populations. This allowed us to directly measure changes in genetic diversity and accumulation of inbreeding in the Arid Recovery populations. We use these results to make recommendations regarding the need for genetic rescue at Arid Recovery and, more broadly, comment on reintroduction strategies that can be used to maintain genetic diversity in small, reintroduced populations

Materials and Methods.

Reintroduction History and Background

The reintroduction history of all four species at Arid Recovery is summarised in Table 1. Reintroductions to Arid Recovery were conducted under ethics approval from the South Australian Wildlife Ethics Committee, approval numbers 42/2005, 6/2005, 19/2000, 22/99, 18/2000, 27/98, 4/99 and 2/2000.

Table 1. Reintroduction and genetic sampling history of the four species translocated to Arid Recovery Reserve (AR). Samples were not available from any of the bilby founding individuals, 32 GSNR founding individuals (12 from Reevesby Island and 18 from Monarto) and the Faure Island founding WBB individuals. We sourced eight WBB samples taken from Faure Island in 2007 as proxies for the AR founders, denoted here with an asterisk. Population size at AR was estimated at the time of sampling from track count data (GSNR, bilbies and WBB) or mark recapture data (bettongs).

Species	**Year of Translocation to AR**	**Number of founders (Male:Female)**	**Source Population(s)**	**Founders sampled at time of release**	**Year of Sampling at AR**	**Samples Collected at AR**	**Population Size at AR (at time of sampling)**
Greater Stick-nest Rat (GSNR)	1998,1999 & 2003	122 (65:57)	Reevesby Island (98), Monarto (24)	Reevesby:84, Monarto:6	2016	20	500
Greater Bilby	2000, 2001 & 2005	37 (21:16)	Monarto (33)	-	2016	16	500
Burrowing Bettong	1999 & 2000	30 (11:19)	Heirisson Prong (10) Bernier (20)	All	2014	60	6000
Western Barred Bandicoot (WBB)	2001 & 2009	17 (4:13)	Bernier Island (12), Faure Island (5)	Bernier:10, Faure:8*	2014	18	1000

A total of 122 (65 male [M] & 57 female [F]) GSNRs were sourced from Reevesby Island (n=98) and a captive breeding colony at Monarto Zoo, South Australia (n=24), and were released at Arid Recovery in 1998 (n=8), 1999 (n=98) and 2003 (n=16) (Moseby and Bice, 2004, Moseby, *pers comm*). Reevesby Island is also a GSNR reintroduction site which was founded using animals from the Monarto captive colony in 1990. The Monarto colony was founded in 1985 by animals sourced from the only remaining natural population of GSNRs on the Franklin Islands, South Australia.

Nine bilbies (3 M & 6 F) were released at Arid Recovery in April 2000. These individuals were sourced from the bilby captive colony at Monarto Zoo, which descends from wild bilbies from Western Australia and the Northern Territory, where natural, remnant populations persist at low density (Moritz *et al.*, 1997; Moseby and O'Donnell, 2003). There are also natural populations of bilbies in Queensland, Australia, but these were not used as source sites for the Arid Recovery reintroductions. A further 28 bilbies from Monarto (n=13: 7 M & 6 F) and Thistle Island (n=15: 11 M & 4 F) were translocated to the reserve between 2001 and 2004. Thistle Island is also a bilby reintroduction site founded from Monarto animals.

A total of 30 (11 M & 19 F) burrowing bettongs were released at Arid Recovery between November 1999 and September 2000 (Moseby *et al.*, 2011). Founding bettongs were sourced from Heirisson Prong (n=10: 3M & 7 F; released in 1999) and the natural, remnant population on Bernier Island (n=20: 8 M & 12 F; released in 2000). Both these sites are in Shark Bay, Western Australia. Heirisson Prong is a reintroduction site whose bettong population was founded from a second remnant population on Dorre Island, also in Shark Bay. The final remnant population of burrowing bettongs, on Barrow Island, Western Australia, was not used for reintroduction to Arid Recovery and is considered by some to be a separate subspecies to the Shark Bay populations (Richards, 2005).

Finally, 12 WBBs (2 M & 10 F), sourced from one of two natural, remnant populations on Bernier Island, were released at Arid Recovery in September 2001. The Arid Recovery WBB population was supplemented in September 2009 when another five WBBs (3 F & 2 M) were translocated from Faure Island, Shark Bay. Faure Island is also a reintroduction site, whose WBB population was founded by individuals from the species' second remnant population on Dorre Island.

Since release, the bettong population at Arid Recovery has increased rapidly with minimal population fluctuations. The WBB population has also increased without substantial bottlenecks, but at a slower rate than the bettongs (Moseby *et al*., 2011). Conversely the bilby and GSNR populations have often fluctuated significantly since release in response to seasonal conditions with populations doubling in size and then crashing to less than 100 individuals during droughts. Population sizes at Arid Recovery at the time of sampling were estimated from track count data for the GSNRs, bilbies and WBBs, and from mark-recapture data for the bettongs (Table 1). As of 2016 there were approximately 500 GSNRs, 500 bilbies, 6000 bettongs, and 1000 WBBs at Arid Recovery (Arid Recovery unpublished data; Moseby, *pers comm*).

Sample Collection:

Founding GSNRs were sampled during their capture on Reevesby Island and at Monarto Zoo in April and November 1999. Founding burrowing bettongs were sampled upon arrival at Arid Recovery from Heirisson Prong in 1999, and on Bernier Island at the point of capture in 2000. Founding WBBs were sampled at the point of capture on Bernier Island in September 2000. Small (2mm) ear tissue samples were taken from founding bettongs and bandicoots using an ear punch, which was swabbed with alcohol prior to and after each use. Stick-nest rat DNA samples from the founders were taken from the tail tip, which was

swabbed, and then 2mm of tissue removed. All founding samples were stored individually and frozen until they were sent to the Australian Biological Tissue Collection (ABTC) at the South Australian Museum, where they were stored at -80°C.

Samples were not taken from the bilby founding individuals, the five WBBs translocated from Faure Island, and 32 of the GSNRs released from Reevesby Island in 1999 (n=12) and Monarto in 1998 (n=2) and 2003 (n=16). We sourced eight WBB ear-clip samples, collected during routine trapping as above and stored in individual ethanol vials, on Faure Island in 2007, and use these as a proxy for the Faure WBB founders.

Post-release DNA samples were obtained during routine Arid Recovery monitoring programs or through targeted trapping and capture opportunities. Arid Recovery WBBs and bettongs were sampled in 2014, while GSNRs and bilbies were sampled in 2016 (Table 1). Animals were captured in large Elliott traps or Sheffield cage traps baited with peanut butter and rolled oats/carrots and apples, or captured using 1.5m long fishing nets and handheld spotlights at night. Ear tissue samples were taken using an ear punch or small sharp scissors and stored in individual vials of ethanol. Samples were stored at -20°C until they were transported to the University of Adelaide. Trapping at Arid Recovery was conducted under an ethics permit from the South Australian Wildlife Ethics Committee (58-2015). The number of samples collected for different populations and species is summarised in Table 1.

DNA Extraction

DNA extraction of tissue samples was performed using a salting out method. Tissue samples stored in ethanol were air dried for 45 minutes prior to extraction before being digested overnight at 55 °C in 300 µL of lysis buffer (10 mM Tris, 0.1 M EDTA pH 8 and 2% SDS), 60 µg of proteinase K, and 0.08 M dithiothreitol (DTT). Digested samples were then incubated at 37 °C with 10 µg of RNase A (Thermo Scientific) for 30 minutes. After

digestion, 100 µL of 7.5 M ammonium acetate was added, the mixture was vortexed and left on ice for an hour. The samples were then centrifuged at 13,000 rpm for 5 minutes and the pellet was discarded. The supernatant was mixed with 300 µl of isopropanol and 10 µg of glycogen (Sigma) and then spun at 15,000 rpm for 10 minutes. The supernatant was discarded and the pellet washed in 300 µL of 70% ethanol and then air dried for 30 minutes. The DNA pellet was resuspended at 65 °C for an hour in 40 µL of TLE buffer (10 mM Tris, 0.1 M EDTA, pH 8). The DNA extracts were quantified using the Quantus Fluorometer system (Promega) as per the manufacturer's instructions.

ddRAD-seq Library Preparation

We generated ddRAD-seq libraries consisting of 95 samples and a library blank following the protocol of Poland *et al*. (2012) with some modifications. Digestion and ligation reactions were performed in 96-well plates. We digested 300 ng of each DNA extract at 37°C for 2 hours using 8 U of the restriction endonucleases *PstI* and *HpaII* in 20 µL of 1x CutSmart Buffer and H_2O (New England Biosciences [NEB]). *PstI* is a rare cutting enzyme with a six-base recognition site (CTGCAG) and *HpaII* is a more common cutting enzyme with a four-base recognition site (CCGG).

Uniquely barcoded adapters (see SI methods and SI Table 1) were ligated to the sticky ends of the digested fragments. Ligation reactions were performed in 40 µL volumes consisting of 20 µl of digested DNA, 200 U of T4 ligase, 0.1 ρmol of forward (rare) and 15 ρmol of reverse (common) adapters (SI Figure 1), 1x T4 Buffer and H_2O. The mixture was left at room temperature for 2 hours, and then heat killed at 65°C for 20 minutes. We pooled the ligation products into 12 libraries of 8 samples each. Pooled libraries were purified using the QIAquick PCR purification kit (Qiagen) and eluted in 120 µL of EB buffer (Qiagen).

PCR reactions to add the full-length Illumina adapters (Poland *et al*., 2012) were performed in 8 replicates per library in 30 µL volumes containing 10 µL of purified library, 1x Hot Start Taq Master Mix (NEB), 0.66 µM each of the forward and reverse primers (SI Figure 1) and H_2O. The PCR conditions were as follows: 95° C for 30 seconds, 16 cycles of 95° C for 30 seconds, 65° C for 20 seconds, and 68° C for 30 seconds, followed by 68° C for 5 minutes, and 25° C for 1 minute. The eight replicates per library were re-pooled and purified as above, eluting in 30 µL of EB buffer (Qiagen). We employed a two-step double-SPRI protocol (Lennon *et al.*, 2010) to select for fragments between 100 and 300 bp using a homemade SPRI bead mix (Rohland and Reich, 2012). Libraries were then quantified using Tapestation 2200 (Agilent) and pooled at equi-molar concentrations. Pooled libraries were sequenced in 1x75 bp (single-end) high output reactions on the Illumina Next-seq at the Australian Genome Research Facility, Adelaide.

Sequence Processing

We used STACKS v1.35 pipeline (Catchen *et al*., 2013, 2011) to process the ddRAD-seq data for each species separately, employing parameters recommended by Mastretta-Yanes *et al.* (2015) to minimise errors and maximise SNP recovery. Raw sequencing reads were de-multiplexed, truncated to 65 bp, and filtered for overall quality based on the presence of barcodes using the *process_radtags* module. Samples with fewer than 500,000 reads were excluded from further analysis. RAD loci were identified for each sample using the *ustacks* module, requiring a minimum stack read depth of three (m=3) and a maximum of two nucleotide mismatches (M=2) between stacks at a locus. Loci with more than three stacks (mls=3) and more reads than two standard deviations above the mean were filtered as they may map to multiple points on the genome. A ‘deleveraging algorithm’ was used to try to resolve over-merged loci. A catalogue of consensus loci among individuals for each species

was constructed with the *cstacks* module using the *ustacks* output files. Loci were recognized as homologous across individuals if they mismatched at two or fewer bases (n=3). Alleles were identified in each individual against this catalogue using the module *sstacks*. The module *populations*, was used to remove potential homologs by filtering out loci with heterozygosity >0.7 and the resulting SNP datasets were output to a PLINK format file (i.e. ped and map files). Finally, the program PLINK (Purcell *et al*., 2007) was used to filter out loci with more than 25% missing data and minor allele frequencies of <0.05. Although removing loci with low minor allele frequencies prohibits tracing the loss of rare alleles in the Arid Recovery populations, we believe this conservative step is necessary to avoid incorporating erroneously called loci.

Quality Control

Raw sequences from blank control samples were also run through the STACKS pipeline, matching the *ustacks* output to the consensus catalogue of all four species. Our aim was to remove any potentially erroneous loci in our datasets that were also present in the library blank samples. However, upon inspection, none of the loci found in the blank controls were present in any of the final datasets, having been filtered at subsequent steps of the pipeline.

A subset of samples from each species was sequenced twice in separate libraries to allow the estimation of error rates. Replicate reads were subsampled to 1 million, 750,000, and 500,000 reads to control for sequencing depth. All subsampled replicates were run through the STACKS pipeline as above, matching the *ustacks* output to the previously constructed consensus catalogue for each species. Allelic error rate was then estimated by counting mismatching alleles at loci for which both replicates had been sequenced.

Genetic Diversity

For each species, samples were grouped by source/descendant population so that comparisons could be made between each founding group and its descendant population at Arid Recovery. For each group we calculated observed and expected heterozygosity (H_O, H_E) using the program GENODIVE v2.0b27 (Meirmans and Van Tienderen, 2004), and allelic richness corrected for sample size (A_R) using the R package *hierfstat* (Goudet, 2005). Individual heterozygosity and inbreeding coefficients (*F*) were calculated in PLINK (Purcell *et al.*, 2007). We tested for significant differences in average individual heterozygosity and *F* between the reintroduced population and their founding groups (where available) using a Wilcoxon rank sum test, corrected for multiple testing.

Wang's pairwise relatedness coefficient (*PR*, Wang, 2002) was estimated for all pairs of individuals within each species using the R package *Related* (Pew *et al.*, 2015). *PR* measures the genetic relatedness or similarity of two individuals relative to the average genetic similarity in the total sample (Hardy, 2003). Consequently, negative values may be obtained if two individuals are less related than the average in the reference.

Temporal Differentiation

PCA, pairwise F_{ST}, sNMF and Bayescan analyses were performed to test for differentiation between the founders and descendants. The bilby dataset did not include founder samples and so was excluded from these analyses

We visualised the variation in our datasets and differentiation between founders and descendants by performing a principal components analysis (PCA) in *adegenet v2.0.1* (Jombart, 2008). PCA is a statistical method for exploring datasets that have a large number of measurements; it reduces the variation in the dataset to a few principal components, which can then be projected onto a graph (Reich *et al*., 2008).

Genetic distance between founding groups (i.e. founders grouped by source population) and descendants was measured as pairwise F_{ST} in Arlequin v3.5. (Excoffier and Lischer, 2010) using the underlying pairwise distance matrix and 10,000 permutations. Significance values were corrected for multiple tests using the Bonferroni correction (Rice, 1989).

We then used the program sNMF v1.2 to estimate the proportional ancestry in each descendant dataset (Frichot *et al*., 2014). Similar to the widely-used program STRUCTURE (Pritchard *et al*., 2000), sNMF estimates the proportion of each individual's genome that originated from a specified number of gene pools (K). Unlike STRUCTURE, sNMF is capable of efficiently analysing large SNP datasets and is more robust to many of the demographic assumptions of Hardy-Weinberg and linkage equilibrium (Frichot *et al.*, 2014). We calculated ancestry proportions in our dataset by running ten replicates of K 1-20 with default parameters and chose the best-supported K as the one with the lowest cross-entropy criterion (CEC), as calculated in sNMF.

We tested for signatures of selection using the F_{ST}-outlier method implemented in Bayescan v2.01 using the default settings (Foll and Gaggiotti, 2008). Bayescan estimates the probability that each locus is subject to selection by teasing apart population-specific and locus-specific components of F-coefficients using a logistic regression. Using a reversible jump Markov chain Monte Carlo (MCMC) algorithm, the posterior probability of a locus being under selection is assessed by testing whether the locus-specific component is necessary to explain the observed pattern of diversity, which infers a departure from neutrality. A threshold value to detect selection was set using a conservative maximum false discovery rate (the expected proportion of false positives) of 0.05.

Population Structure Within Arid Recovery

We also tested for population differentiation and structuring within the Arid Recovery Reserve. The reserve is divided into 6 fenced paddocks, four of which (Northern Expansion, First Expansion, Second Expansion and the Main Exclosure) have reintroduced animals within them (Figure 1). Although some animals are known to move through, over or under the fences between paddocks, we wanted to test whether the fencing was discouraging gene flow. We used PCA, pairwise F_{ST} and sNMF analyses as above, but using only the contemporary descendent population samples, grouped by the paddock in which they were sampled.

Results

Sequencing Results

We successfully sequenced 95 GSNR, 15 bilby, 71 bettong and 35 WBB samples, (summarised in Table 2 and SI Table 2), generating a large SNP dataset (1752-8703 SNPs) for each species. The WBB samples yielded fewer SNPs (n=1752) than the other species, despite similar sequencing success and locus discovery, suggesting lower average genetic diversity in this species. This is in agreement with previous studies showing very low genetic diversity in WBBs using microsatellite, mitochondrial (Smith and Hughs, 2008), and MHC (Smith *et al*., 2010) markers.

The average estimated allelic error rates, calculated between pairs of replicates subsampled to varying depths for each species was 1.2-6.6%, as shown in SI Table 3-6. The error rate did not differ with sequencing depth for any species indicating that our cut-off of 500,000 reads per sample was appropriate.

Table 2. Summary of sequencing statistics for each founder group and descendent population within each species.

Species	Population	Number of Samples successfully sequenced	Average Number of Reads	Average Number of Loci	Average Depth of Coverage	Number of SNPs in final dataset	Average Missing Data
Greater stick-nest rat	Reevesby Island	72	4148368.65	142615.26	20.66	8703	11.90%
Greater stick-nest rat	Monarto	6	2545679.00	110442.33	16.36	8703	15.80%
Greater stick-nest rat	Arid Recovery	17	4428737.94	158270.65	20.55	8703	6.80%
Greater bilby	Arid Recovery	15	5597898.73	97196.40	38.54	6880	13.23%
Burrowing bettong	Bernier Island	18	2427230.28	55023.33	32.76	3775	10.50%
Burrowing bettong	Heirisson Prong	6	748519.83	27520.17	20.45	3775	28.30%
Burrowing bettong	Arid Recovery	47	2633766.28	52221.19	35.50	3775	9.40%
Western barred bandicoot	Bernier Island	9	4775200.44	71154.11	44.71	1752	13.80%
Western barred bandicoot	Faure Island	8	2480600.38	69239.75	26.11	1752	11.70%
Western barred bandicoot	Arid Recovery	18	3821004.94	66350.67	41.68	1752	8.60%

Genetic Diversity and Inbreeding

Observed heterozygosity across all groups (i.e. Arid Recovery and source populations) ranged from 0.14 to 0.31 and was lower than expected heterozygosity under Hardy-Weinberg equilibrium (HWE) for all populations except for the Faure Island WBBs (Table 3). Allelic richness ranged from 1.13 (Faure Island WBBs) to 1.34 (Arid Recovery bettongs). The WBBs had the lowest genetic diversity of the four species, again consistent with previous studies (Smith and Hughes, 2008; Smith *et* al., 2010).

Table 3. Average measures of genetic diversity in founding and descendant populations of mammals released at Arid Recovery, with standard deviation in parentheses. Allelic richness corrected for sample size (A_R), and expected and observed heterozygosity (H_E, H_O).

Species	**Population**	**H_E**	**H_O**	**A_R**
Greater stick-nest rat	Reevesby Island	0.33 (0.001)	0.31 (0.001)	1.33 (0.14)
Greater stick-nest rat	Monarto	0.33 (0.002)	0.30 (0.003)	1.32 (0.21)
Greater stick-nest rat	Arid Recovery	0.30 (0.002)	0.29 (0.002)	1.30 (0.17)
Greater bilby	Arid Recovery	0.31 (0.002)	0.26 (0.002)	1.28 (0.16)
Burrowing bettong	Bernier Island	0.21 (0.003)	0.20 (0.004)	1.21 (0.21)
Burrowing bettong	Heirisson Prong	0.23 (0.004)	0.18 (0.004)	1.22 (0.26)
Burrowing bettong	Arid Recovery	0.34 (0.002)	0.31 (0.002)	1.34 (0.13)
Western barred bandicoot	Bernier Island	0.15 (0.002)	0.14 (0.002)	1.15 (0.19)
Western barred bandicoot	Faure Island	0.13 (0.005)	0.15 (0.006)	1.13 (0.21)
Western barred bandicoot	Arid Recovery	0.24 (0.003)	0.21 (0.003)	1.24 (1.33)

The bettongs and WBBs at Arid Recovery had higher diversity across all measures, than either of their founding groups. On the other hand, the Arid Recovery GSNR population

had slightly lower diversity across all measures than their founders. Although we could not do similar comparisons with the bilby dataset, as founding samples were not available, we note that their diversity measures are similar to the other species at Arid Recovery.

We further explored genetic diversity by calculating individual heterozygosity (Figure 2). Average individual heterozygosity was significantly higher in the Arid Recovery bettongs compared to its two founding populations ($p<0.05$), while all other comparisons between populations or groups were non-significant ($p>0.05$). The distribution of individual heterozygoisty within groups of all species demonstrates how genetic diversity is relatively even across individuals within each population, except for within the Arid Recovery WBBs. In this group, five individuals are much more heterozygous than all other samples. Most individuals in the WBB population have lower heterozygosity than the founding group, but the average has been driven up by the five outliers.

Figure 2. Individual observed heterozygosity calculated for each sampled individual of greater stick-nest rat (GSNR), greater bilby, burrowing bettong and western barred bandicoot (WBB). Each vertical bar represents an individual, and is coloured by population. Population names have been shortened: A.R —Arid Recovery; R.I. — Reevesby Island; Mo. — Monarto; H.P. — Heirisson Prong; B.I. — Bernier Island; F.I. — Faure Island.

The Arid Recovery bettong and WBB populations' average inbreeding were lower than either of their founding groups (Figure 3). However, only the bettong population had significantly different average inbreeding compared to their founders ($p<0.05$). The WBB inbreeding was highly variable, with most sampled individuals having higher coefficients than the founders. The five Arid Recovery WBB individuals with high heterozygosity, and therefore, much lower inbreeding coefficients than the rest of the WBB group again drove this pattern. The Arid Recovery GSNR population had slightly higher (although non-significantly, $p>0.05$) average inbreeding than either of their founding groups, and the Arid Recovery bilby population had comparable average inbreeding to the Arid Recovery GSNR and bettong populations..

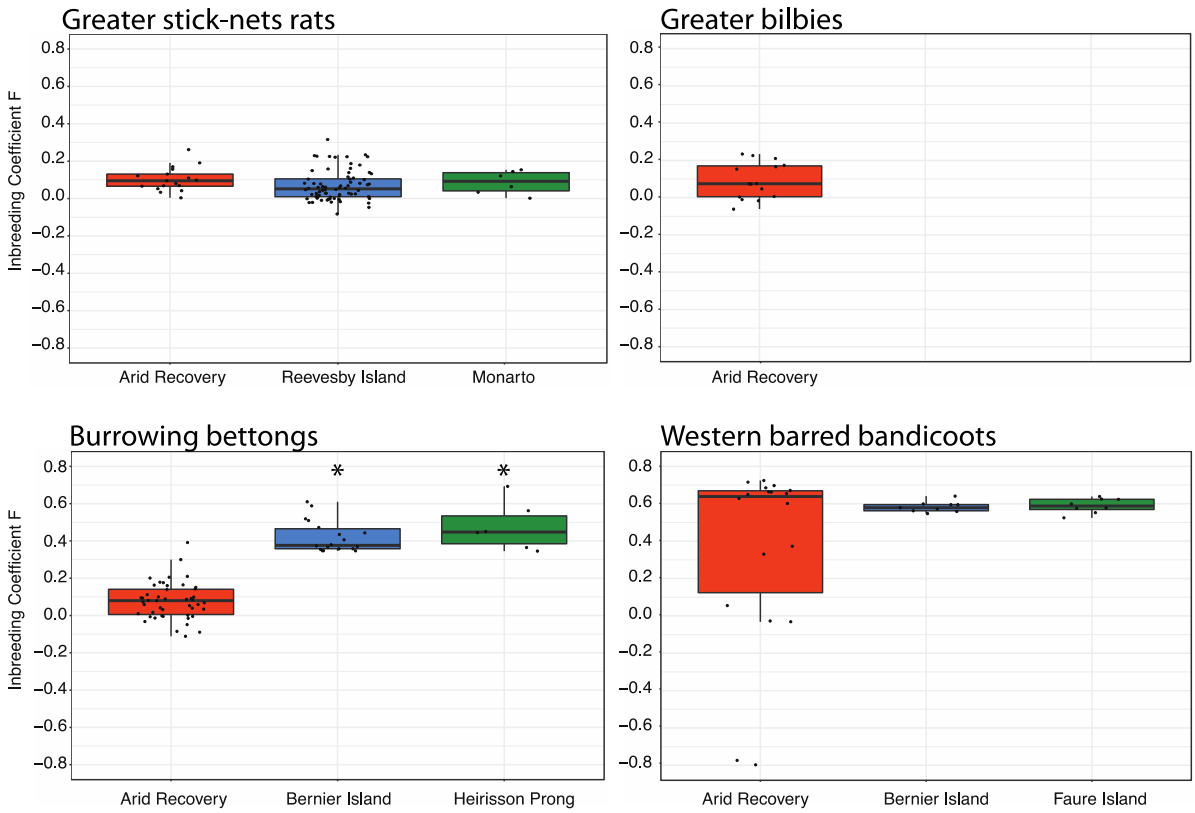

Figure 3. Individual inbreeding coefficients per population for founding groups (where available) and current Arid Recovery populations of greater stick-nest rats (GSNR), greater bilbies, burrowing bettongs and western barred bandicoots (WBB). Dots represent individual values. Middle horizontal lines represent the median, the boxes are bound by the 25th and 75th quartiles and vertical lines show the minimum and maximum range of values excluding outliers. Founding groups that had significantly different average inbreeding coefficients from their descendant Arid Recovery populations are denoted with an asterisk

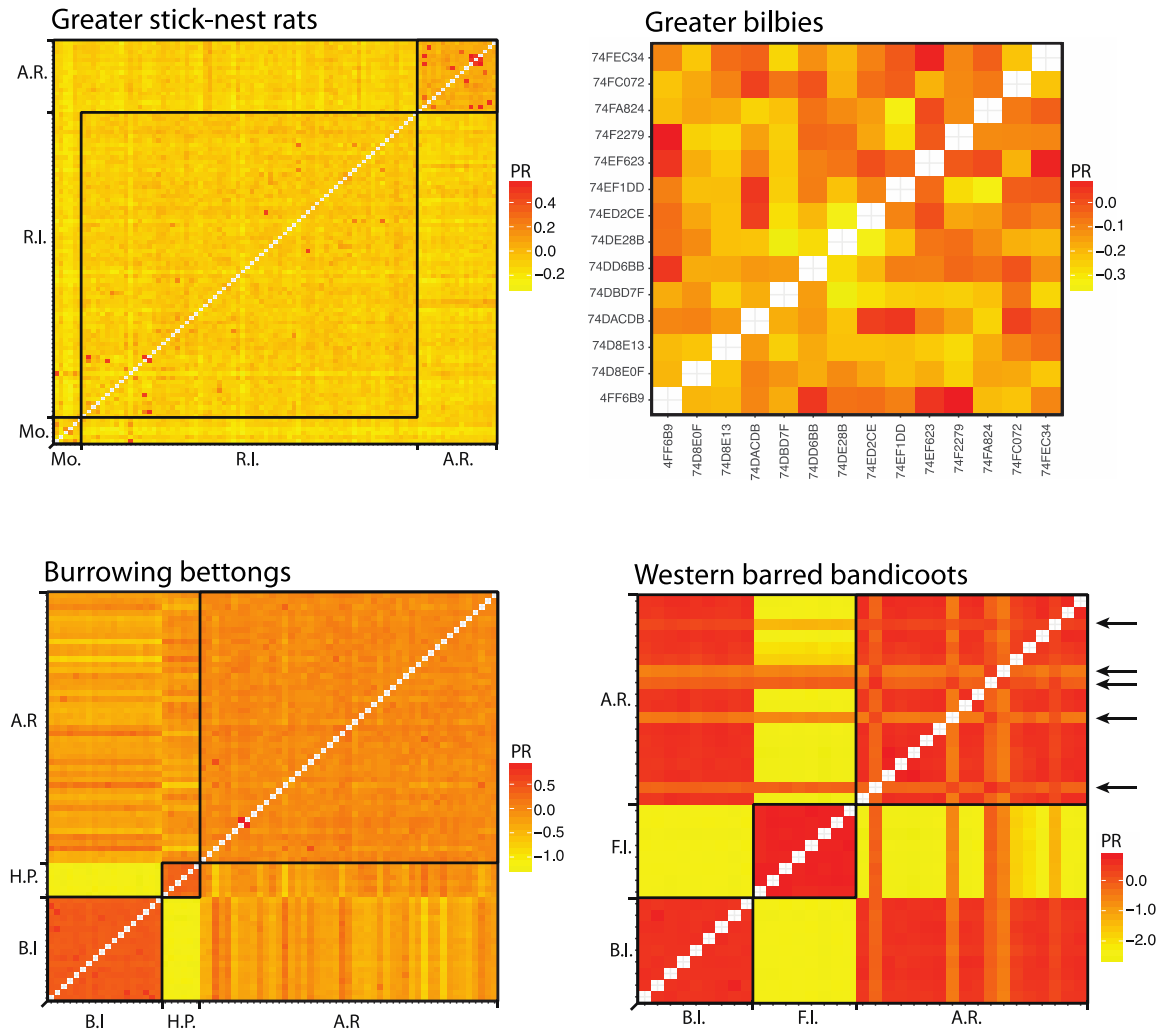

Figure 4. Heat map of pairwise relatedness (*PR*) calculated between each sampled individual within each species. Within population comparisons are bounded by black squares. Arrows on the WBB heat map highlight the five individuals with lower levels of inbreeding and average pairwise relatedness than the rest of the WBB Arid Recovery samples. Population names are shortened due to space requirements: A.R — Arid Recovery; R.I. — Reevesby Island; Mo. — Monarto; H.P. — Heirisson Prong; B.I. — Bernier Island; F.I. — Faure Island. Bilby PR is labelled by sample as founding individuals were not sampled

Average *PR* between individuals was higher within the Arid Recovery GSNR population than in either of its founding groups (Figure 4). Conversely, average *PR* was

lower in the bettong and WBB Arid Recovery populations compared to their founding groups (Figure 4). However, the *PR* in the WBBs was again quite varied, and lowest between the same five individuals that also had lower inbreeding and higher heterozygosity. The *PR* measured in the bettong and WBB populations also show that the two founding groups for each species (Bernier Island and Heirisson Prong in bettongs, and Bernier Island and Faure Island for the WBBs), were highly unrelated to each other and that the WBB Arid Recovery population was more related to its Bernier Island founding group than the Faure Island founding group, excepting the five outlier individuals, which were equally related to both founding groups. *PR* within the Arid Recovery bilby population was varied, but generally low.

Arid Recovery Differentiation from Founding Groups

The results of principle component analysis for the GSNR, bettong and WBB datasets are shown in Figure 5. The GSNR Arid Recovery population is identifiable as a cluster separate from both founding groups of Monarto and Reevesby Island individuals, although the total amount of variation explained by the first two principle components is low (2.24-3.4%). The Arid Recovery bettong population clusters as a group intermediate between its two founding groups, Bernier Island and Heirisson Prong. Finally, the Arid Recovery WBB samples cluster with its Bernier Island founding group separate to the Faure Island proxy founders. The five WBB individuals with lower inbreeding and higher heterozygosity are the most intermediate between the rest of the Arid Recovery/Bernier Island group and the Faure Island cluster.

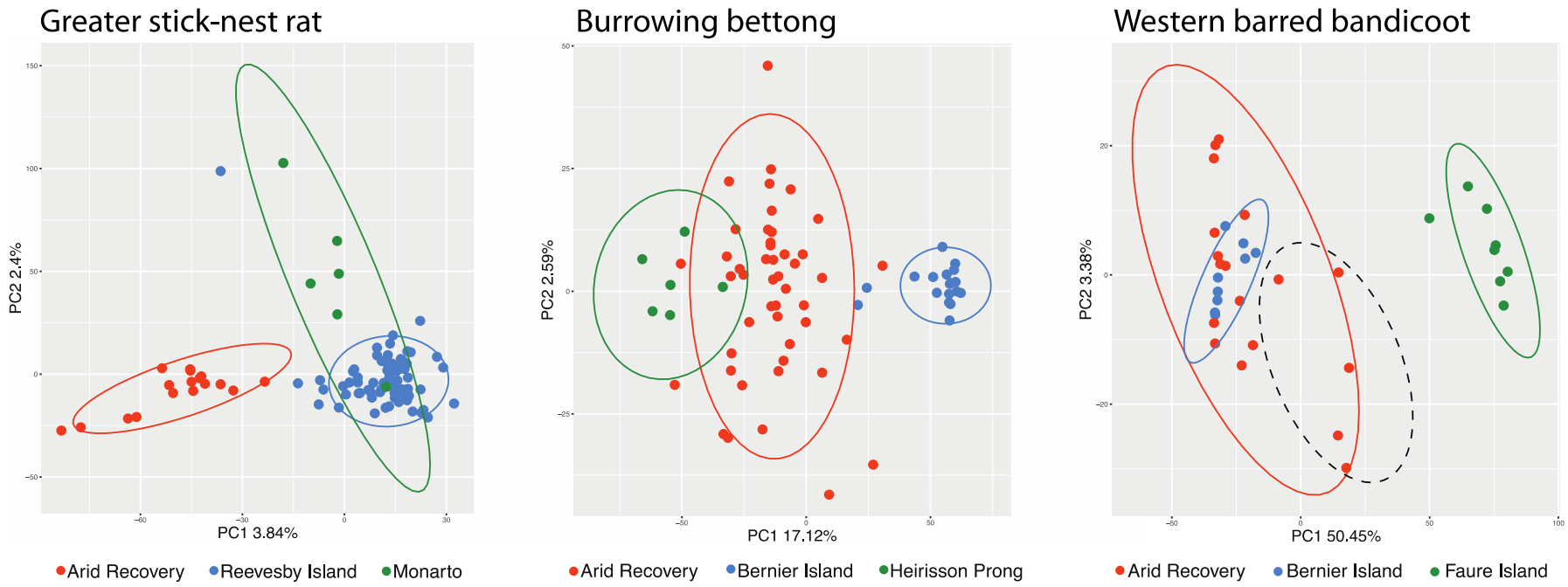

Figure 5. Relationships among founding groups and the descendant Arid Recovery populations of greater stick-nest rats (GSNR), burrowing bettongs and western barred bandicoots (WBB) based on principle coordinate analysis for principle components 1 and 2. Each dot represents an individual coloured by population. Solid ellipses represent the centre and 95% confidence interval of the points in each population. The dotted ellipse encompasses the five outlier WBB samples.

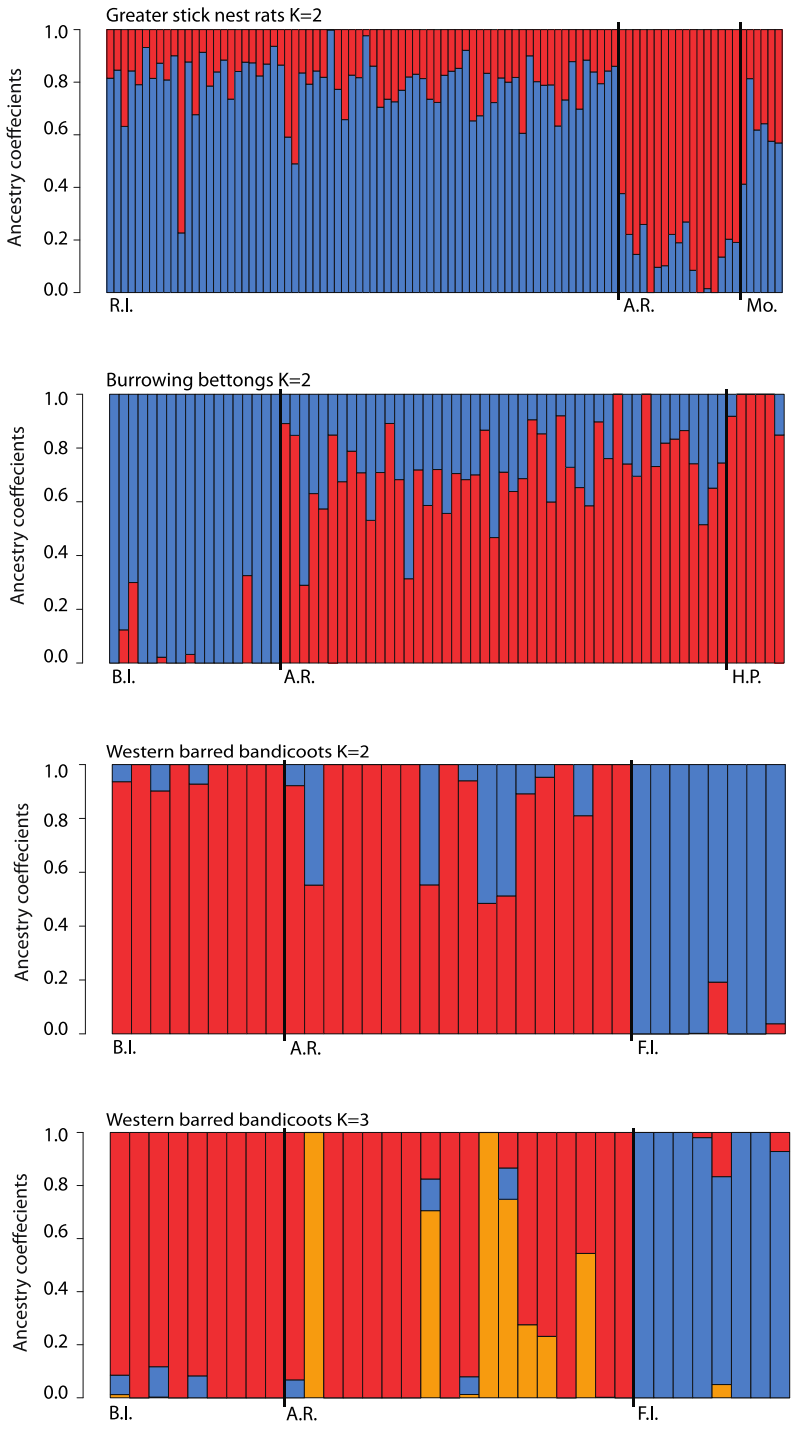

Figure 6. Genetic ancestry in individuals from Arid Recovery and their founding groups estimated using sNMF. Each vertical bar represents an individual. Population names are shortened due to space requirements: A.R — Arid Recovery; R.I. — Reevesby Island; Mo. — Monarto; H.P. — Heirisson Prong; B.I. — Bernier Island; F.I. — Faure Island.

Pairwise F_{ST} values are shown in Table 4 and are in general agreement to the PCA results. F_{ST} values between GSNR groups were significantly different from zero between Arid Recovery and the founding groups, but not between the Monarto and Reevesby Island animals. All pairwise F_{ST} values were significantly different from zero between all groups of bettongs, being highest between the two founding groups (Heirisson Prong and Bernier Island). Within the WBB dataset, pairwise F_{ST} was significantly different from zero between Arid Recovery and the Faure Island group, and between the two founding groups (Faure Island and Bernier Island), but not between Bernier Island and Arid Recovery.

Table 4. Pairwise F_{ST} values calculated between the founding groups and descendant Arid Recovery populations for the greater stick-nest rats, burrowing bettongs and western barred bandicoots. Significant values (after Bonferroni correction) are highlighted in bold.

Greater stick-nest rats (GSNR)

	Arid Recovery	**Reevesby Island**	**Monarto**
Arid Recovery			
Reevesby Island	**0.04352**		
Monarto	**0.05930**	0.02845	

Burrowing bettongs

	Arid Recovery	**Bernier Island**	**Heirisson Prong**
Arid Recovery			
Bernier Island	**0.19133**		
Heirisson Prong	**0.11992**	**0.53907**	

Western barred bandicoots (WBB)

	Arid Recovery	**Bernier Island**	**Faure Island**
Arid Recovery			
Bernier Island	0.03933		
Faure Island	**0.67165**	**0.8124**	

The sNMF analysis inferred that the most likely number of ancestral gene pools was two for the GSNR and bettong datasets, and three for the WBB dataset (SI Figure 2). Results of the ancestry estimates are shown in Figure 6. The GSNR plot shows most individuals in this dataset are a mixture of two genepools, with Reevesby Island dominated by one (average of 80% 'blue' in the plot) and Arid Recovery dominated by the other (average of 85% 'red' in the plot). The bettong sNMF plot shows that the Arid Recovery population is a mixture of the Bernier Island (mainly all blue) and Heirisson Prong (mainly all red) founders with an average of 71% Heirisson Prong and 29% Bernier Island ancestry.

The WBB sNMF plot shows that most Arid Recovery individuals share their entire ancestry with the Bernier Island founders. However, seven individuals are estimated (under K=3) to have ancestry from a third source (shown in orange on the plot). When we plot the ancestry estimates for the WBB dataset under K=2 (as the known number of sources, Figure 6) we can see that those seven individuals are those with admixture from the Faure Island population. We also note that the five individuals with the most Faure Island ancestry correspond to the individuals that were found to be the least inbred and most heterozygous.

Bayescan analysis identified six loci under putative selection in the GSNR dataset, but none in the bettong or WBB datasets (SI Figure 3). These six loci represents 0.07% of the total GSNR dataset and had FST values of >0.19 compared to an average of 0.05 across all loci.

Population Structure Within Arid Recovery

We did not detect any significant population structuring within Arid Recovery reserve for any of the sampled species. The PCA plots (SI Figure 4) show near panmixia of the Arid Recovery population in all species, although a number of GSNR individuals cluster closely together on both axes. The WBB PCA shows a large amount of variation within Arid

Recovery in PC 1, but this is driven by the five individuals with the most Faure Island admixture, rather than by structuring within the reserve. In agreement with the within Arid Recovery PCA results (SI Figure 4), none of the pairwise F_{ST} comparisons between paddocks for any species was significantly different from zero after correction for multiple testing (SI Table 7). Finally, sNMF analysis identified the most likely number of gene pools within Arid Recovery for the GSNR, bettongs and bilbies as one (indicating no structuring) and two for the WBBs (SI Figure 5). The sNMF plot of the Arid Recovery WBB population again identifies the Faure Island admixture, which is evenly spread between the paddocks (SI Figure 6).

Discussion

Change in Genetic Diversity Since Release at Arid Recovery Reserve

Despite relatively small founding populations, but perhaps consistent with modest-to-large population growth in all four species over an ~18-year period, our results show that average genetic diversity in all populations of reintroduced, threatened mammals at Arid Recovery reserve are close to, or exceeding, the levels measured in their founding groups. We detect only a small reduction in genetic diversity and small increase in inbreeding since release in the GSNR population, while the bettong and WBB populations are, on average, more diverse and less inbred than their founding groups. This result is driven by the mixing of two diverged and individually inbred source populations, which has had a large positive impact on the genetic diversity of the descendant Arid Recovery population. Our study suggests that additional translocations to Arid Recovery may not necessary at this time, and

highlights the power of admixture, even from small isolated populations, as a tool for conservation management to maximise genetic diversity in threatened taxa via genetic rescue.

GSNRs at Arid Recovery have retained between 94 and 98% of genetic diversity (depending on the measure used) and show no significant increase in inbreeding compared to their founding groups. These results indicate that most of the genetic diversity captured in the founding individuals from Monarto and Reevesby Island has been retained in the Arid Recovery populations, possibly because of the larger-than-average number of founders released (n = 122).

However, we do detect a small amount of differentiation between the GSNR Arid Recovery population and their founding groups, indicated by the small, but significant, pairwise F_{ST} values, and both the sNMF analysis and PCA plot. This differentiation could be due to selection. For example, unlike the other populations of reintroduced species, the Arid Recovery GSNR population experiences high mortality due to heat stress during summer, which may be acting as a selective pressure in this population (Moseby, *pers comm*). This hypothesis is partially supported by our Bayescan analysis, which detected six loci under putative selection in the GSNR dataset. However, F_{ST} outliers can also result from demographic effects, such as wave-edge surfing in recently bottlenecked populations (Hofer *et al.*, 2009; Klopfstein *et al.*, 2006). Given the probable small effective population size in the Arid Recovery population that would limit natural selection (Frankham *et al.*, 2010), genetic drift is a more likely explanation for the differentiation seen in the GSNRs here. Further field experiments comparing fitness of locally sourced and translocated animals in the Arid Recovery environment could be used to test the hypothesis of local adaptation in the Arid Recovery population. Such research is crucial to understanding how drift and selection can be differentiated and ultimately how either case should be treated in translocated populations, particularly when animals are moved between climatic zones.

The bettong and WBB populations have increased average genetic diversity compared to their founding populations. Allelic richness has increased in both populations by more than 7% and measures of heterozygosity have increased between 40% and 80%. We found that in both species these results were entirely driven by admixture between two diverged sources.

Within the Arid Recovery bettong population, this admixture was evenly distributed, likely reflecting the fact that the two groups of founding individuals (from Bernier Island and Heirisson Prong) were released within a year of each other and have had 16 years to interbreed. It is interesting that the majority of ancestry (as shown in the sNMF analysis) in the bettong population was from the Heirisson Prong founders, despite only 10 individuals being released from this source compared to 20 from Bernier Island. This may be due to the additional year that the Heirisson Prong founders had to acclimatize to the new habitat before the Bernier Island founders were released, potentially giving the first group an advantage over the second. Although, this pattern could also be driven by stochastic drift.

Within the WBB Arid Recovery population, the admixture is less evenly distributed than in the bettong population, likely because of the smaller number of individuals translocated from the second source, and the shorter time since second release. Only five individuals were translocated from Faure Island in 2009 (eight years after the first release from Bernier Island), but their genetic impact on the population is clear. Individuals without Faure Island admixture were slightly more inbred and less genetically diverse than the founding groups, whilst the individuals with admixture had much lower inbreeding and much higher heterozygosity than any other sampled individual. The five outlier individuals had roughly half of their ancestry, as estimated by sNMF analysis, originating from Faure Island which indicates they may be F1 hybrids. The Faure Island WBBs released into Arid Recovery were first contained within a pen and allowed to breed with each other before being released into the wider reserve. Given that WBBs live for three to five years, sampling of F1

hybrids is possible. Under the relatively complete panmixia seen within all reintroduced Arid Recovery populations, we expect this admixture in the WBBs to spread throughout the population in subsequent generations. However, to ensure the introgressed genetic diversity is not lost through stochastic processes, the genetic composition of the WBB population should be retested in a biologically relevant time-frame (for example 5-10 generations).

The pattern of admixture in the WBBs compared to that observed in the Arid Recovery bettongs suggest that, where possible, translocation programs should aim to mix a similar number of individuals from different genetic stock simultaneously and early on in the establishment of reintroduced populations to maximise the benefits of admixture on genetic diversity.

The bilby population at Arid Recovery had similar levels of inbreeding and genetic diversity to the GSNR and bettong populations within the reserve. We were, however, unable to assess how much inbreeding had accumulated or how much genetic diversity has been retained since release as samples from the bilby founders were not available. We emphasize the importance of collecting samples from founders during reintroduction programs for use in later genetic assessments, even when individuals are sourced from captive breeding facilities with studbooks. Genotyping samples from other extant populations of bilbies across Australia would improve our inference about how resilient this population is to genetic deterioration. Mortiz *et al*. (1997) examined genetic diversity across the wild bilby range using mitochondrial DNA and microsatellites. Repeating this analysis using SNP data would permit direct comparison with our dataset here and allow recommendations on the need for additional translocations to be made.

Given that our results show that Arid Recovery Reserve has been successful in maintaining or even increasing the genetic diversity in the species reintroduced there, we suggest that additional reintroductions may not be necessary at this time. However, we note

that our datasets did not allow us to detect the true impact of founder effects on the Arid Recovery populations. A founder effect is the reduction of genetic diversity in a new population compared to its source resulting from non-representative founding individuals (i.e. when not all genetic diversity present in a source population is 'captured' in the founding individuals; Frankham *et al*., 2010). We would expect this affect to be exacerbated when serial founder events occur (i.e. when the founding source is itself a reintroduced or captive population), as is the case for some of the Arid Recovery species. Further sampling at source, the original source populations (in the case of serial founding events), and other remnant populations of each species should be prioritised to determine whether genetic diversity can be further increased in the Arid Recovery populations.

Admixture as a Conservation Tool

A significant finding in this study is the positive impact that admixture has had on genetic diversity in two of the reintroduced mammal populations at Arid Recovery. The impact of admixture and gene flow on genetic diversity is well established. Wright (1931) and Franklin (1980) estimated that just one migrant per generation would be enough to prevent population differentiation, drift and loss of adaptive potential (although more recent work suggests 1-10 migrants per generation may be necessary to stop loss of diversity in wild populations; Mills and Allendorf, 1996). Admixture of diverged populations was found to substantially increase the genetic diversity in reintroduced populations of the peregrine falcon (*Falco peregrinus*; Jacobsen *et al*., 2008) and Alpine ibex (*Capra ibex*; Biebach and Keller, 2012), even when divergence between the source populations was low. Furthermore, genetic rescue (i.e. deliberate introduction of individuals from other populations to restore genetic diversity and fitness) is an effective strategy to increase the reproductive health of small,

inbred populations (Heber *et al*., 2013; Hedrick and Fredrickson, 2010; Madsen *et al*., 2004; Weeks *et al.*, 2015).

Despite the evident advantages, admixture has been underutilized as a conservation tool due to concerns about outbreeding depression and the need to conserve locally adapted variation within subpopulations (Frankham, 2015; Weeks *et al*., 2016, 2011). However, outbreeding depression is unlikely when mixing animals from populations that share similar environments, have the same karyotype, have previously exchange genes and/or have long generation times (Frankham *et al*., 2010). Furthermore, Weeks *et al*. (2016) argue that many populations previously perceived as genetically 'unique' and potentially locally adapted using neutral genetic markers, are often more likely to have differentiated through random genetic drift and are therefore the populations most likely to be in need of genetic restoration.

The source populations of the WBBs and bettongs at Arid Recovery are from similar environments, all originating from islands in Shark Bay, Western Australia, and are therefore unlikely to have different local adaptations. Additionally, a recent study found only minor mitochondrial haplotype divergence between the two WBB remnant populations (Smith and Hughes, 2008). Hence, the admixture at Arid Recovery is unlikely to have resulted in outbreeding depression. Rather, the bettong population at Arid Recovery, which was admixed from the outset of the reintroduction program, has seen the most significant population growth of all the reintroduced species at the reserve, suggesting a possible fitness advantage in the admixed animals. Further experiments examining the fitness levels of inbred compared to outbred/admixed bettongs is needed to test this hypothesis. Regardless of whether this admixture confers any fitness advantages in the Arid Recovery populations, mixing of the diverged source populations will contribute to the preservation of adaptive potential in these species.

Conclusion

Our high-resolution datasets have revealed the success of the Arid Recovery reintroduction programs in maintaining and maximising genetic diversity of the threatened mammal species released there. Our results suggest that additional translocations to Arid Recovery may be unnecessary at this time, and highlight the clear benefit to reintroduction programs of admixing slightly diverged populations to maximise genetic diversity and adaptive potential in threatened taxa. Comparison of the two admixture strategies employed in the bettong and WBB populations at Arid Recovery show that future translocation programs that plan to mix different genetic stocks should aim to release equal numbers of animals from both sources simultaneously, early in the reintroduction program. This will promote balanced admixture of both sources in the descendant population.

Ultimately, we have demonstrated the benefits of genetic monitoring in reintroduction programs and advocate for its continued use at Arid Recovery and in other reintroduction programs in the future.

Data Availability: All de-multiplexed raw sequencing data are available from NCBI's short read archive (Accession number: PRJNAXXXXXX).

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Supplementary Materials

Design and Preparations of Barcoded Adapters

Both the barcoded forward primer and the common reverse primer (a Y-adapter) were designed as per Poland *et al*. (2012; see SI Figure 1). A set of 96 barcodes were designed using the barcode-generator python script (https://github.com/audy/barcode-generator) to range in size from 5-9 bp in length with a Levenstein distance of at least 3 to allow samples to be distinguished from one another even with one sequencing error in each barcode (see SI methods). The single stranded oligonucleotides of each barcode adapter and the common adapter were resuspended to 100 μM in 1x Elution Buffer (EB; 10mM Tris-Cl, pH=8.0). To make a plate of working aliquots for the double stranded adapters, we added 10 μl of each single stranded oligo (at 100 μM) to 10 μl of 10x Adapter Buffer (AB; 500mM NaCl, 100mM Tris-Cl) and 70 μl of H_2O. This mixture was then heated to 95° C for 2 minutes, and cooled at 1° C per minute until 30° C was reached, and then held at 4° C for 5 minutes. The barcoded adapters were then diluted 3:10 with AB and quantified using Quant-iT Picogreen dsDNA dye (Invitrogen) on a Quantus fluorometer (Promega Corporation). Each barcoded adapter was normalised to 1.6 ng/μl (=0.1μM). A plate containing a combination of the forward barcoded adapter and common reverse adapter was then prepared by adding 20 μl of the barcoded adapter (at 0.1 μM) to 30 μl of the common reverse adapter (at 10 μM) and 50 μl of 1x AB.

SI Figure 1. Configuration of barcoded adapters and full length Illumina sequencing adapters.

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SI Table 1: Unique barcodes used for each double-digest RAD library. Shown here in 96-well plate format.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	CTCGAGT	GTGTGAGT	GAGTATG	GACTCT	AGACGAGA	CCGTCGCCA	GCAGA	CGAGAG	TCCGCCGCA	GCTCG	ACGATG	GTACGT
B	AGCACTA	TGCATGCGT	CATGGCCGA	CTATCG	GAACAAGT	ACACT	TGATCTA	AGGCGACCT	ACCTACCG	GTATTCTAT	CAGCAGA	GCCAT
C	GTCGATG	TCACGAGT	AATGGACA	ACTCGCCA	TCAGATG	TGGCAA	ATGTACT	CCGCGTCCA	ATCACTAT	ATCAGAG	CTCAT	TACTGATAG
D	GTCGTACTG	ATGGCG	CGTATGCT	CGAGCGT	TGCCACCA	TACTACAG	CCGGACTGA	GAGCGTCGT	TACGA	ATACACAG	CTGAAGAA	TTCCGA
E	TCTCTCA	CGTGATGCA	CACTGAG	TGCAGCG	AGTGACAA	ATCTGTCT	GTCTG	CAGTATAGA	TAGATGTA	GCATCAGCG	CATCCG	ATTCGG
F	TATGATTCT	TAGCGTG	AGCAGTCT	TCTTATAGT	GTACGCTGT	CTATA	TCACTG	GGCTAG	ATGTCA	GTGTGCAT	ACTACGTG	CGTCGA
G	CCGATGGCA	CACTCG	TGTATAT	TTGTTAACT	ACCTG	TATCT	AGTATAGT	TGTAG	TCATGG	TTAATCGTT	CGCGCATA	CTGCG
H	AGCTA	GTGCGATAG	ACGTGTACT	ACTCTA	GCTGACG	ACGAGTG	ATCTGTA	TGCTAT	TCGTACTA	CAGATA	TAGGCT	AGCCGTGCA

SI Table 2: Sequencing details of all samples used in this study. The five outlier WBB samples mentioned in the main text are denoted with an asterisk.

Sample	Species	Pop	Sex	Reads	No. of Loci	Average Depth of Coverage	No. of SNPs	Missing Data
282	Leporillus conditor	Arid Recovery	female	2624274	116537	16.74	7214	17.11%
284	Leporillus conditor	Arid Recovery	female	2722355	116790	17.12	7979	8.32%
294	Leporillus conditor	Arid Recovery	female	5189201	170074	24.50	8480	2.56%
321	Leporillus conditor	Arid Recovery	female	1747771	91034	11.99	6207	28.68%
328	Leporillus conditor	Arid Recovery	N/A	3699057	148902	18.50	8243	5.29%
347	Leporillus conditor	Arid Recovery	female	3576200	136011	19.74	7940	8.77%
430	Leporillus conditor	Arid Recovery	male	4361914	154409	22.20	8289	4.76%
435	Leporillus conditor	Arid Recovery	N/A	2663562	112107	16.39	7890	9.34%
493	Leporillus conditor	Arid Recovery	N/A	6622181	211613	24.48	8580	1.41%
513	Leporillus conditor	Arid Recovery	female	8031913	201222	32.80	8604	1.14%
516	Leporillus conditor	Arid Recovery	male	8386909	234469	29.24	8620	0.95%
530	Leporillus conditor	Arid Recovery	male	4380176	168614	19.86	8344	4.13%
559	Leporillus conditor	Arid Recovery	male	3724920	156128	17.88	8295	4.69%
560	Leporillus conditor	Arid Recovery	N/A	3471059	144980	17.73	8171	6.11%
597	Leporillus conditor	Arid Recovery	N/A	6077728	204073	23.62	8597	1.22%
646	Leporillus conditor	Arid Recovery	male	4750963	174597	21.01	8507	2.25%
2197	Leporillus conditor	Arid Recovery	N/A	3258362	149041	15.48	7655	12.04%
6001	Leporillus conditor	Reevesby Island	female	3562713	138711	19.02	7841	9.90%
6002	Leporillus conditor	Reevesby Island	male	4822016	168566	21.76	8503	2.30%
6003	Leporillus conditor	Reevesby Island	female	2720249	115613	17.31	7501	13.81%
6004	Leporillus conditor	Reevesby Island	female	6874160	196716	27.51	8583	1.38%
6006	Leporillus conditor	Reevesby Island	male	2121320	106741	13.81	7183	17.47%
6007	Leporillus conditor	Reevesby Island	male	11527763	279400	33.60	8630	0.84%

6009	Leporillus conditor	Reevesby Island	male	3933136	146984	20.70	8142	6.45%
6010	Leporillus conditor	Reevesby Island	male	7235658	194627	29.59	8501	2.32%
6011	Leporillus conditor	Reevesby Island	female	1810268	80475	15.24	6878	20.97%
6014	Leporillus conditor	Reevesby Island	female	6516891	191091	27.35	8466	2.72%
6015	Leporillus conditor	Reevesby Island	female	5279965	162513	25.43	8312	4.49%
6016	Leporillus conditor	Reevesby Island	female	4429305	162149	20.65	8504	2.29%
6017	Leporillus conditor	Reevesby Island	female	1374737	70029	12.52	5635	35.25%
6018	Leporillus conditor	Reevesby Island	female	2512222	109692	15.73	7896	9.27%
6019	Leporillus conditor	Reevesby Island	female	1109242	57961	10.78	5043	42.05%
6020	Leporillus conditor	Reevesby Island	female	2146368	101834	13.94	7537	13.40%
6021	Leporillus conditor	Reevesby Island	female	3342776	143707	17.25	8062	7.37%
6022	Leporillus conditor	Reevesby Island	female	5063856	170874	22.66	8349	4.07%
6023	Leporillus conditor	Reevesby Island	male	4735540	173311	21.18	8511	2.21%
6024	Leporillus conditor	Reevesby Island	female	3830691	135381	20.86	8221	5.54%
6026	Leporillus conditor	Reevesby Island	male	5361895	179446	23.68	8566	1.57%
6027	Leporillus conditor	Reevesby Island	female	6672776	193934	27.24	8597	1.22%
6030	Leporillus conditor	Reevesby Island	male	5978084	174431	27.29	8533	1.95%
6031	Leporillus conditor	Reevesby Island	male	2657781	121889	15.92	7647	12.13%
6033	Leporillus conditor	Reevesby Island	male	4395750	151217	21.95	8322	4.38%
6035	Leporillus conditor	Reevesby Island	female	4338173	161604	20.17	8438	3.04%
6036	Leporillus conditor	Reevesby Island	male	2727863	116844	16.46	8031	7.72%
6037	Leporillus conditor	Reevesby Island	male	3306814	143647	16.80	8122	6.68%
6038	Leporillus conditor	Reevesby Island	female	2980655	123523	17.31	8031	7.72%
6039	Leporillus conditor	Reevesby Island	male	2776634	116126	16.98	7281	16.34%
6040	Leporillus conditor	Reevesby Island	female	4844842	169300	22.51	8472	2.65%
6041	Leporillus conditor	Reevesby Island	male	3502000	140178	18.81	8165	6.18%
6042	Leporillus conditor	Reevesby Island	female	5846226	186009	24.93	8555	1.70%
6043	Leporillus conditor	Reevesby Island	female	7014082	194629	28.91	8586	1.34%
6301	Leporillus conditor	Reevesby Island	male	5957349	175793	26.90	8523	2.07%

6302	Leporillus conditor	Reevesby Island	male	7946053	201326	31.85	8561	1.63%
6303	Leporillus conditor	Reevesby Island	male	5164334	165274	24.60	8454	2.86%
6304	Leporillus conditor	Reevesby Island	male	2439437	98427	18.19	7475	14.11%
6305	Leporillus conditor	Reevesby Island	male	1341994	71600	11.86	5147	40.86%
6306	Leporillus conditor	Reevesby Island	female	962517	45656	13.14	3975	54.33%
6308	Leporillus conditor	Reevesby Island	male	3418055	119844	21.97	7194	17.34%
6309	Leporillus conditor	Reevesby Island	male	3664626	140247	19.65	7266	16.51%
6310	Leporillus conditor	Reevesby Island	male	4343418	141167	24.12	7697	11.56%
6311	Leporillus conditor	Reevesby Island	male	5743541	185296	24.09	8398	3.50%
6312	Leporillus conditor	Reevesby Island	male	6712318	217950	24.44	8596	1.23%
6313	Leporillus conditor	Reevesby Island	female	1552939	71955	13.79	5463	37.23%
6314	Leporillus conditor	Reevesby Island	female	2629099	130001	14.28	7551	13.24%
6315	Leporillus conditor	Reevesby Island	female	5797846	201079	22.50	8570	1.53%
6317	Leporillus conditor	Reevesby Island	male	5069803	179199	22.32	8560	1.64%
6318	Leporillus conditor	Reevesby Island	female	3749760	140996	19.90	8250	5.21%
6319	Leporillus conditor	Reevesby Island	female	3504089	128088	20.72	7238	16.83%
6320	Leporillus conditor	Reevesby Island	female	5991492	189046	25.09	8569	1.54%
6322	Leporillus conditor	Reevesby Island	female	3147220	127573	18.25	7619	12.46%
6323	Leporillus conditor	Reevesby Island	female	5776631	192029	23.65	8578	1.44%
6324	Leporillus conditor	Reevesby Island	female	928256	41942	13.79	3613	58.49%
6325	Leporillus conditor	Reevesby Island	male	2430544	112683	15.26	7244	16.76%
6326	Leporillus conditor	Reevesby Island	male	3506182	129863	19.93	7803	10.34%
6327	Leporillus conditor	Reevesby Island	female	2752424	118017	16.71	7471	14.16%
6328	Leporillus conditor	Reevesby Island	male	1668713	85246	13.07	6017	30.86%
6329	Leporillus conditor	Reevesby Island	female	5241695	164683	25.72	8373	3.79%
6330	Leporillus conditor	Reevesby Island	female	4670781	134939	27.20	8116	6.74%
6331	Leporillus conditor	Reevesby Island	female	1323380	69248	12.15	6011	30.93%
6332	Leporillus conditor	Reevesby Island	male	2229417	105942	14.73	6993	19.65%
6333	Leporillus conditor	Reevesby Island	male	5485231	176274	24.95	8341	4.16%

6334	Leporillus conditor	Reevesby Island	female	8585082	229603	29.68	8624	0.91%
6335	Leporillus conditor	Reevesby Island	male	4463745	160044	21.05	8377	3.75%
6337	Leporillus conditor	Reevesby Island	female	1492285	65974	15.40	4198	51.76%
6338	Leporillus conditor	Reevesby Island	female	2264804	91728	17.94	6735	22.61%
6339	Leporillus conditor	Reevesby Island	male	5186259	166083	24.62	8322	4.38%
6340	Leporillus conditor	Reevesby Island	female	9380477	210428	36.50	8418	3.27%
6341	Leporillus conditor	Reevesby Island	N/A	3221365	122277	19.19	8130	6.58%
609A	Leporillus conditor	Reevesby Island	female	1586931	81627	12.57	5735	34.10%
6044	Leporillus conditor	Monarto	female	4005091	144880	21.21	8189	5.91%
6045	Leporillus conditor	Monarto	female	2710666	121883	16.00	7842	9.89%
6046	Leporillus conditor	Monarto	female	2361385	109678	15.49	7165	17.67%
6047	Leporillus conditor	Monarto	male	1796858	90768	13.57	6317	27.42%
6048	Leporillus conditor	Monarto	female	2142067	102406	14.54	6959	20.04%
6049	Leporillus conditor	Monarto	male	2258007	93039	17.36	7472	14.14%
4FF6B9	Macrotis lagotis	Arid Recovery	N/A	703252	32567	14.27	3626	47.30%
74D8E0F	Macrotis lagotis	Arid Recovery	female	1295592	54863	16.92	5093	25.97%
74D8E13	Macrotis lagotis	Arid Recovery	female	785303	34664	15.19	4180	39.24%
74DACDB	Macrotis lagotis	Arid Recovery	female	1230020	53435	16.50	4946	28.11%
74DBD7F	Macrotis lagotis	Arid Recovery	N/A	4566371	96200	39.05	6644	3.43%
74DD6BB	Macrotis lagotis	Arid Recovery	female	4403483	87806	40.70	6266	8.92%
74DE28B	Macrotis lagotis	Arid Recovery	female	9239086	140837	55.52	6764	1.69%
74ED2CE	Macrotis lagotis	Arid Recovery	female	1594029	56289	21.21	5321	22.66%
74EEE4D	Macrotis lagotis	Arid Recovery	female	20078702	188661	90.85	6750	1.89%
74EF1DD	Macrotis lagotis	Arid Recovery	female	6430009	125196	42.21	6783	1.41%
74EF623	Macrotis lagotis	Arid Recovery	female	4347325	105185	33.94	6646	3.40%
74F2279	Macrotis lagotis	Arid Recovery	female	7286569	127473	47.58	6761	1.73%
74FA824	Macrotis lagotis	Arid Recovery	male	12798264	154769	70.63	6768	1.63%
74FC072	Macrotis lagotis	Arid Recovery	female	3027573	79423	30.68	6219	9.61%
74FEC34	Macrotis lagotis	Arid Recovery	female	6182903	120578	42.94	6774	1.54%

16292	Bettongia lesueur	Arid Recovery	male	6654467	96781	59.84	3733	1.11%
16293	Bettongia lesueur	Arid Recovery	male	4176948	79555	44.82	3729	1.22%
16294	Bettongia lesueur	Arid Recovery	N/A	3566192	76056	40.31	3747	0.74%
16295	Bettongia lesueur	Arid Recovery	male	3672767	76958	40.26	3730	1.19%
16296	Bettongia lesueur	Arid Recovery	male	6447357	91286	61.31	3735	1.06%
16297	Bettongia lesueur	Arid Recovery	male	6799944	94024	62.52	3742	0.87%
16298	Bettongia lesueur	Arid Recovery	male	280465	13624	13.50	1911	49.38%
16299	Bettongia lesueur	Arid Recovery	female	2276350	42628	43.22	3694	2.15%
16301	Bettongia lesueur	Arid Recovery	female	551257	21367	18.87	2984	20.95%
16303	Bettongia lesueur	Arid Recovery	female	3225819	48552	56.15	3682	2.46%
16304	Bettongia lesueur	Arid Recovery	male	1765499	38132	37.53	3611	4.34%
16305	Bettongia lesueur	Arid Recovery	female	2312328	43019	43.66	3695	2.12%
16306	Bettongia lesueur	Arid Recovery	female	1279374	29864	33.10	3461	8.32%
16307	Bettongia lesueur	Arid Recovery	male	1707273	38085	35.49	3624	4.00%
16309	Bettongia lesueur	Arid Recovery	female	702655	24298	22.12	3212	14.91%
16310	Bettongia lesueur	Arid Recovery	N/A	1110179	30899	27.58	3524	6.65%
16311	Bettongia lesueur	Arid Recovery	male	1642981	38006	34.72	3634	3.74%
16312	Bettongia lesueur	Arid Recovery	male	471661	18428	18.18	2812	25.51%
16313	Bettongia lesueur	Arid Recovery	male	1076340	31131	26.89	3474	7.97%
16315	Bettongia lesueur	Arid Recovery	male	463850	18152	17.54	2740	27.42%
16319	Bettongia lesueur	Arid Recovery	female	668858	23436	20.90	3237	14.25%
16320	Bettongia lesueur	Arid Recovery	male	3321175	52542	52.32	3743	0.85%
16321	Bettongia lesueur	Arid Recovery	female	1500810	37325	31.73	3666	2.89%
16322	Bettongia lesueur	Arid Recovery	female	3630341	54839	55.80	3734	1.09%
16323	Bettongia lesueur	Arid Recovery	male	244344	11011	13.07	1814	51.95%
16327	Bettongia lesueur	Arid Recovery	female	339841	14401	15.50	2196	41.83%
16328	Bettongia lesueur	Arid Recovery	male	10104970	111260	79.38	3706	1.83%
16329	Bettongia lesueur	Arid Recovery	female	3009386	70462	36.19	3732	1.14%
16330	Bettongia lesueur	Arid Recovery	female	590859	23716	17.74	2387	36.77%

16331	Bettongia lesueur	Arid Recovery	female	3281541	72102	38.27	3738	0.98%
16332	Bettongia lesueur	Arid Recovery	male	7087400	99157	61.53	3726	1.30%
16333	Bettongia lesueur	Arid Recovery	male	714033	30712	16.65	3054	19.10%
16334	Bettongia lesueur	Arid Recovery	male	499109	23298	13.76	2601	31.10%
16335	Bettongia lesueur	Arid Recovery	female	2028421	56554	29.26	3700	1.99%
16336	Bettongia lesueur	Arid Recovery	male	4201009	67048	52.30	3729	1.22%
16338	Bettongia lesueur	Arid Recovery	male	6963109	92804	64.48	3732	1.14%
16340	Bettongia lesueur	Arid Recovery	male	1252032	42688	23.08	3549	5.99%
16341	Bettongia lesueur	Arid Recovery	female	1002954	36963	20.87	3027	19.81%
16342	Bettongia lesueur	Arid Recovery	male	2433345	62818	32.17	3730	1.19%
16343	Bettongia lesueur	Arid Recovery	male	1390395	46096	23.86	3475	7.95%
16344	Bettongia lesueur	Arid Recovery	female	4090655	81822	43.17	3728	1.25%
16345	Bettongia lesueur	Arid Recovery	male	1081349	41187	20.13	3663	2.97%
16346	Bettongia lesueur	Arid Recovery	male	3772281	80952	40.39	3749	0.69%
16347	Bettongia lesueur	Arid Recovery	female	2491464	63489	32.63	3719	1.48%
16348	Bettongia lesueur	Arid Recovery	female	2013311	60883	27.29	3693	2.17%
16350	Bettongia lesueur	Arid Recovery	male	2715700	70608	32.51	3736	1.03%
16351	Bettongia lesueur	Arid Recovery	male	3174617	75378	35.69	3726	1.30%
15953	Bettongia lesueur	Bernier Island	female	1464363	46600	25.21	3688	2.30%
15954	Bettongia lesueur	Bernier Island	male	371874	17656	13.85	2783	26.28%
15955	Bettongia lesueur	Bernier Island	female	363030	17220	13.40	1703	54.89%
15956	Bettongia lesueur	Bernier Island	female	2918045	71895	34.74	3723	1.38%
15957	Bettongia lesueur	Bernier Island	female	2193779	63578	28.83	3727	1.27%
15959	Bettongia lesueur	Bernier Island	male	1144946	43164	21.07	3357	11.07%
15960	Bettongia lesueur	Bernier Island	female	5398927	88497	52.46	3733	1.11%
15961	Bettongia lesueur	Bernier Island	male	4323248	82096	44.91	3739	0.95%
15962	Bettongia lesueur	Bernier Island	female	1126932	43044	20.53	3342	11.47%
15963	Bettongia lesueur	Bernier Island	female	1975957	44072	35.83	3620	4.11%
15965	Bettongia lesueur	Bernier Island	male	1540726	51621	23.97	3690	2.25%

15966	Bettongia lesueur	Bernier Island	female	3534783	77113	39.32	3740	0.93%
15967	Bettongia lesueur	Bernier Island	female	1134242	40032	22.14	3701	1.96%
15968	Bettongia lesueur	Bernier Island	male	1460418	43415	26.90	3732	1.14%
15969	Bettongia lesueur	Bernier Island	female	549551	9065	47.50	1394	63.07%
15970	Bettongia lesueur	Bernier Island	male	6087092	89642	58.30	3718	1.51%
15971	Bettongia lesueur	Bernier Island	female	2065807	59256	29.16	3690	2.25%
15972	Bettongia lesueur	Bernier Island	male	6036425	102454	51.64	3739	0.95%
15947	Bettongia lesueur	Heirisson Prong	female	894940	28938	24.57	2573	31.84%
15951	Bettongia lesueur	Heirisson Prong	N/A	709466	26440	20.06	3083	18.33%
15952	Bettongia lesueur	Heirisson Prong	female	885191	33065	20.77	2764	26.78%
15973	Bettongia lesueur	Heirisson Prong	male	974851	36736	20.15	3262	13.59%
15975	Bettongia lesueur	Heirisson Prong	female	544654	20338	19.34	2795	25.96%
15976	Bettongia lesueur	Heirisson Prong	female	482017	19604	17.81	1750	53.64%
15977	Perameles_bougainville	Bernier_Island	female	970410	31317	22.94	1223	30.18%
15978	Perameles_bougainville	Bernier_Island	female	5355250	93625	48.98	1727	1.43%
15979	Perameles_bougainville	Bernier_Island	female	735491	28963	17.93	995	43.18%
15981	Perameles_bougainville	Bernier_Island	female	9364540	116727	69.73	1717	2.00%
15982	Perameles_bougainville	Bernier_Island	female	811927	29164	19.97	1265	27.78%
15983	Perameles_bougainville	Bernier_Island	female	3895523	77819	41.06	1706	2.62%
15984	Perameles_bougainville	Bernier_Island	female	12658483	121986	90.74	1710	2.40%
15985	Perameles_bougainville	Bernier_Island	female	1241837	37138	24.85	1518	13.35%
15986	Perameles_bougainville	Bernier_Island	male	7943343	103648	66.21	1725	1.54%
16285	Perameles_bougainville	Arid_Recovery	N/A	704824	24670	20.36	1141	34.85%
16287*	Perameles_bougainville	Arid_Recovery	N/A	1198504	34700	26.18	1358	22.48%
16288	Perameles_bougainville	Arid_Recovery	N/A	2318265	47878	38.99	1678	4.22%
16289	Perameles_bougainville	Arid_Recovery	N/A	1752917	41822	33.66	1604	8.44%
16290	Perameles_bougainville	Arid_Recovery	N/A	1771382	42885	32.56	1632	6.85%
16291	Perameles_bougainville	Arid_Recovery	N/A	2318054	47206	38.52	1664	5.02%
16715	Perameles_bougainville	Arid_Recovery	female	6195402	101793	52.43	1720	1.83%

16716*	Perameles_bougainville	Arid_Recovery	male	8906476	107136	72.84	1709	2.45%
16717	Perameles_bougainville	Arid_Recovery	N/A	8653573	106094	72.05	1724	1.60%
16718	Perameles_bougainville	Arid_Recovery	male	853085	31416	19.97	1355	22.65%
16719*	Perameles_bougainville	Arid_Recovery	male	2118870	59296	27.78	1632	6.85%
16720*	Perameles_bougainville	Arid_Recovery	male	10090622	110614	80.42	1694	3.31%
16721	Perameles_bougainville	Arid_Recovery	male	7915264	102083	68.32	1714	2.17%
16722	Perameles_bougainville	Arid_Recovery	female	3995465	74554	46.27	1637	6.56%
16723	Perameles_bougainville	Arid_Recovery	female	4153141	80558	44.36	1710	2.40%
16724*	Perameles_bougainville	Arid_Recovery	male	2178366	66167	27.01	1625	7.24%
16725	Perameles_bougainville	Arid_Recovery	male	2488406	70444	29.14	1685	3.82%
16726	Perameles_bougainville	Arid_Recovery	male	1165473	44996	19.44	1532	12.55%
WBB03	Perameles_bougainville	Faure_Island	female	3089214	81213	30.60	1702	2.85%
WBB04	Perameles_bougainville	Faure_Island	male	4932881	101041	40.79	1712	2.28%
WBB07	Perameles_bougainville	Faure_Island	male	2952799	84104	28.04	1692	3.42%
WBB12	Perameles_bougainville	Faure_Island	male	1976200	62452	24.37	1602	8.56%
WBB21	Perameles_bougainville	Faure_Island	N/A	704667	30004	14.76	1142	34.80%
WBB22	Perameles_bougainville	Faure_Island	N/A	1632687	57556	20.85	1602	8.56%
WBB72	Perameles_bougainville	Faure_Island	male	3085291	79826	31.02	1649	5.88%
WBB89	Perameles_bougainville	Faure_Island	male	1471064	57722	18.47	1430	18.37%

SI Table 3: Average allelic error rate calculated from four replicated greater stick-nest rat samples.

		Number of reads subsampled in replicate A		
		1 Million	**750k**	**500k**
Number of reads subsampled in replicate B	**1 Million**	0.028	0.027	0.028
	750k	0.027	0.026	0.026
	500k	0.029	0.028	0.028

SI Table 4: Average allelic error rate calculated from five replicated greater bilby samples.

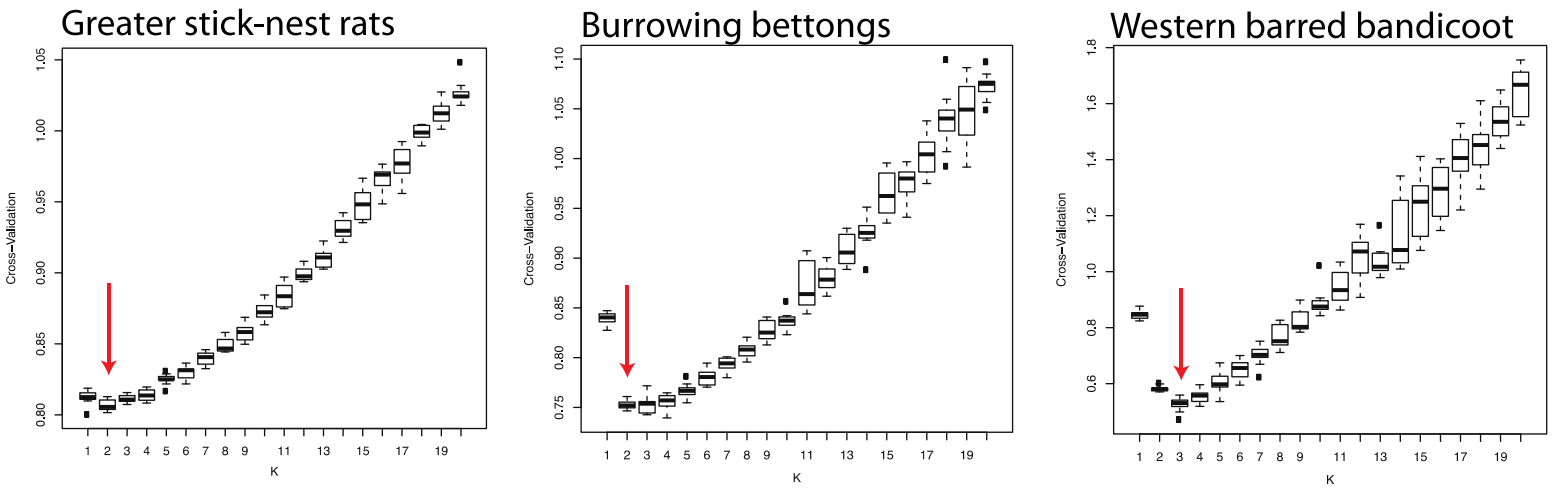
		Number of reads subsampled in replicate A		
		1 Million	**750k**	**500k**
Number of reads subsampled in replicate B	**1 Million**	0.019	0.019	0.020
	750k	0.018	0.019	0.020
	500k	0.019	0.021	0.020

SI Table 5: Average allelic error rate calculated from 12 replicated burrowing bettong samples.

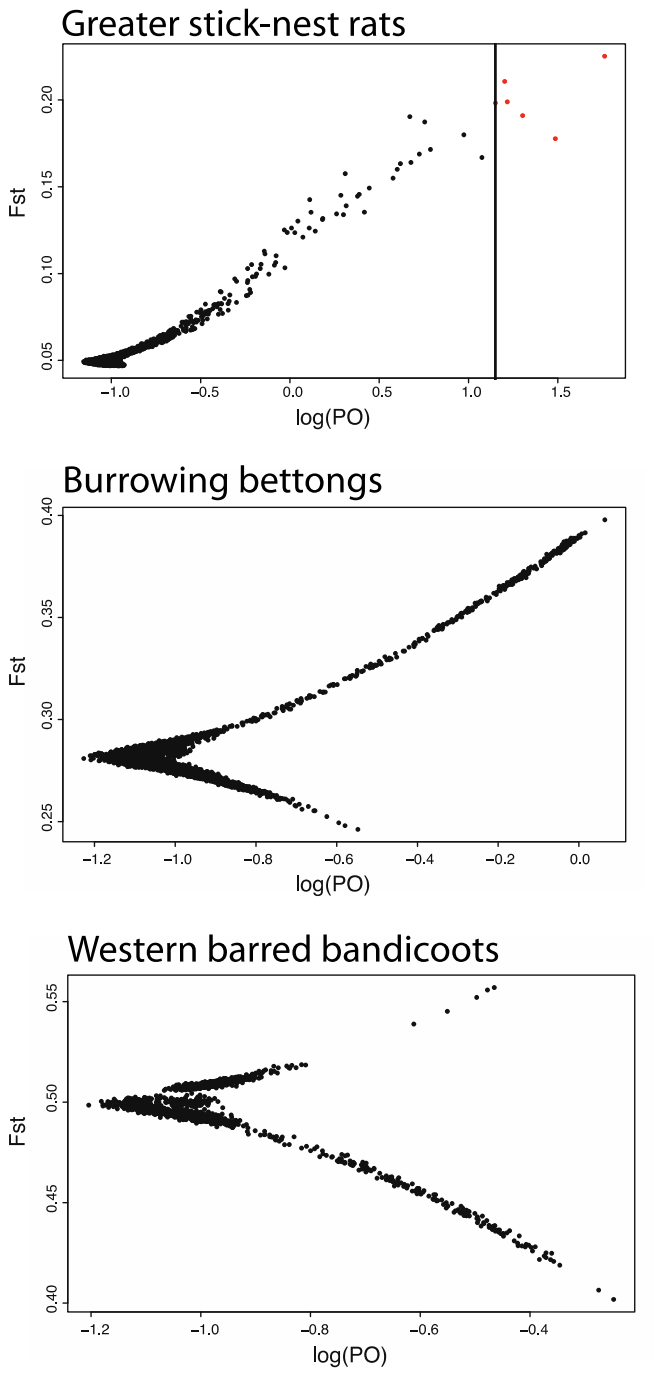
		Number of reads subsampled in replicate A		
		1 Million	**750k**	**500k**
Number of reads subsampled in replicate B	**1 Million**	0.014	0.013	0.012
	750k	0.014	0.013	0.012
	500k	0.014	0.013	0.012

SI Table 6: Average allelic error rate calculated from 10 replicated western barred bandicoot samples.

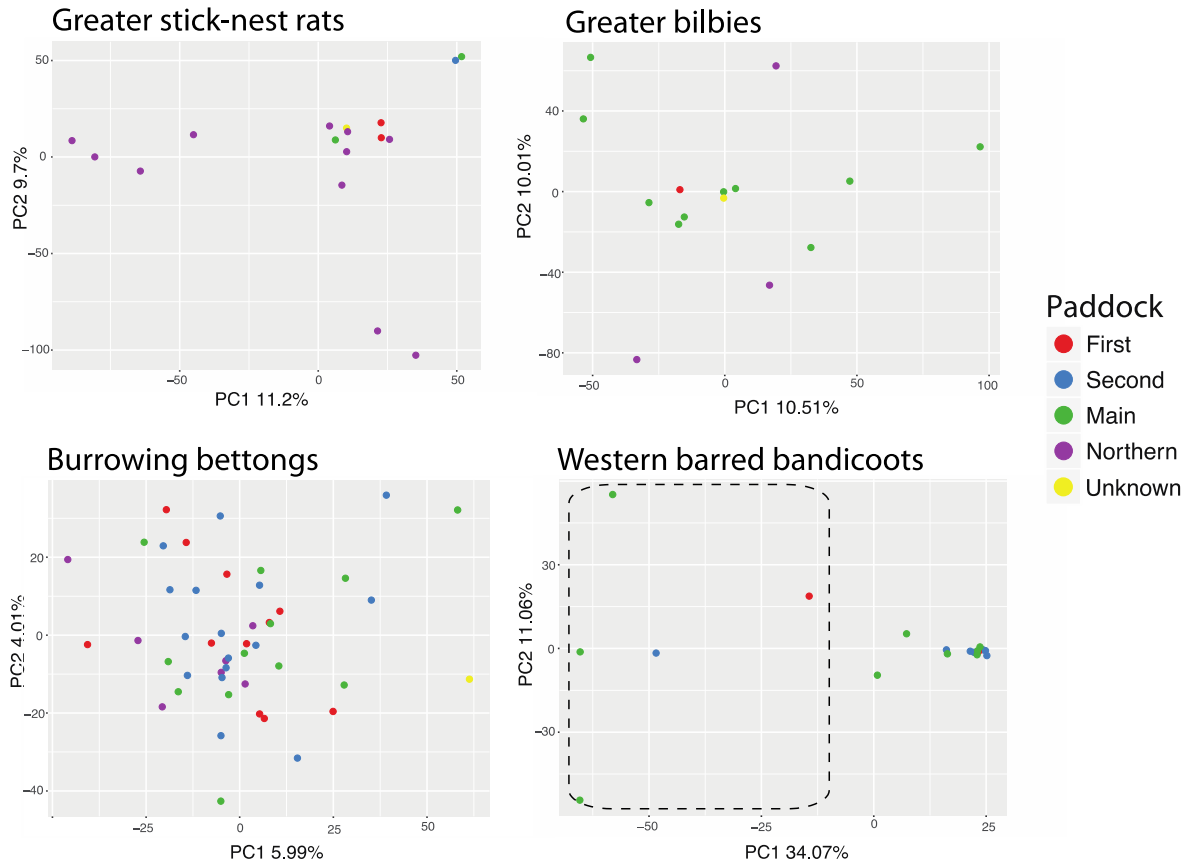
		Number of reads subsampled in replicate A		
		1 Million	**750k**	**500k**
Number of reads subsampled in replicate B	**1 Million**	0.062	0.065	0.065
	750k	0.062	0.061	0.061
	500k	0.066	0.066	0.061



SI Figure 2. sNMF cross-entropy criterion plot. Middle horizontal lines represent the median, the boxes are bound by the 25th and 75th quartiles and vertical lines show the minimum and maximum range of values excluding outliers. K with the lowest median CEC value is taken as the most likely number of 'gene pools' (K) and is highlighted here with the red arrows.



SI Figure 3. Signatures of selection in the greater stick-nest rat, burrowing bettong and western barred bandicoot datasets inferred using the program Bayescan. Each dot represents a locus. The vertical axis indicates mean FST between the Arid Recovery and founding groups and the horizontal axis indicates the log posterior odds (PO). The vertical line indicates the false discovery rate threshold of 0.05 (not shown in the bettong or WBB plots as it is out of range). Loci to the right of this line (red dots) are putatively under selection



SI Figure 4. Relationships among individual greater stick-nest rats, greater bilbies, burrowing bettongs and western barred bandicoots sampled at Arid Recovery reserve based on Principle Coordinate Analysis for principle components 1 and 2. Each dot represents an individual coloured by the paddock in which it was sampled. The dotted ellipse encompasses the five outlier WBB samples with the most Faure Island admixture.

SI Table 7. Pairwise F_{ST} values calculated between samples collected in different paddocks at Arid Recovery for the greater stick-nest rats, greater bilbies, burrowing bettongs and western barred bandicoots. No values were significantly different from zero after correction for multiple testing.

Greater stick-nest rats

	Main	First	Second	Northern
Main				
First	0.05732			
Second	0.02923	0.05276		

Greater bilbies

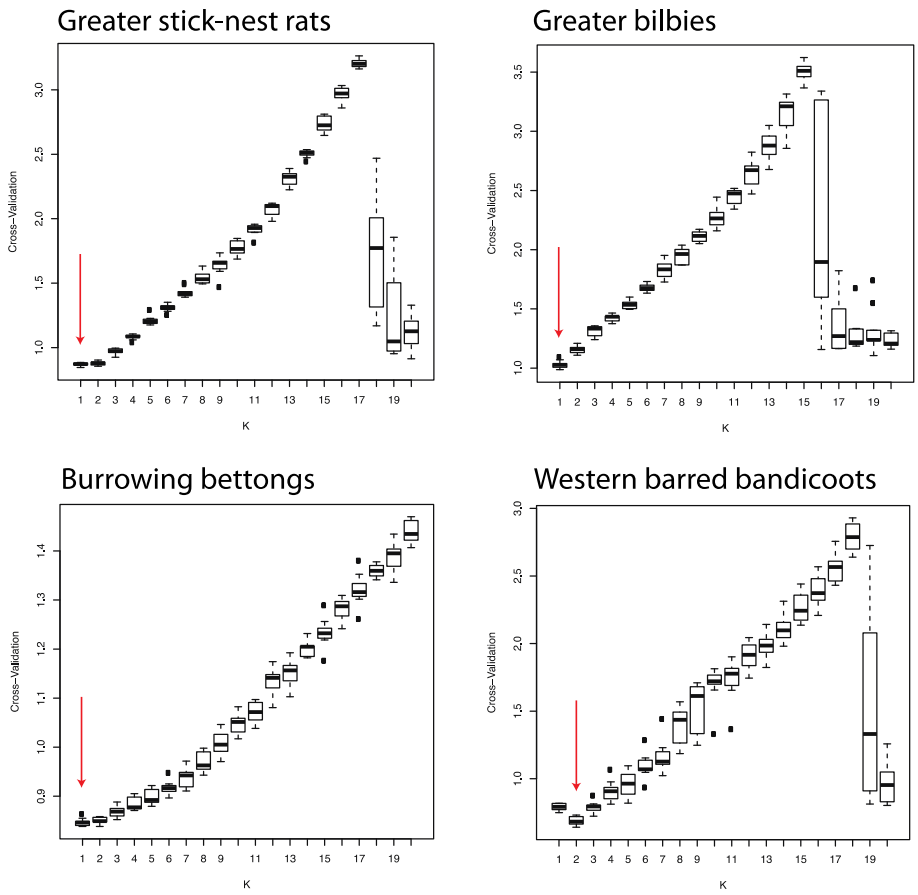
	Main	First	Northern
Main			
First	0.00304		
Northern	0	0.01559	

Burrowing bettongs

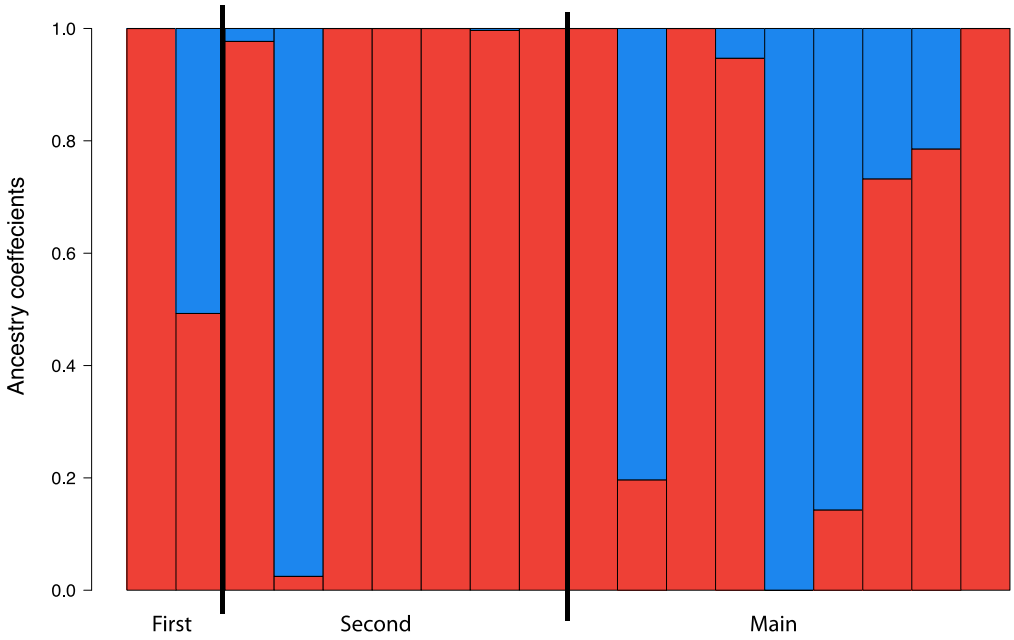
	Main	First	Second	Northern
Main				
First	0.00015			
Second	0	0		
Northern	0.01629	0.01405	0.01055	

Western barred bandicoots

	Main	First	Second
Main			
First	0		
Second	0.04534	0	



SI Figure 5. sNMF cross-entropy criterion plot calculated with the Arid Recovery samples only. Middle horizontal lines represent the median, the boxes are bound by the 25th and 75th quartiles and vertical lines show the minimum and maximum range of values excluding outliers. K with the lowest median CEC value is taken as the most likely number of 'gene pools' (K) and is highlighted here with the red arrows



SI Figure 6. Genetic ancestry in WBB individuals from Arid Recovery estimated using sNMF. Each vertical bar represents an individual, grouped by the paddock in which they were sampled.

Chapter 6

High-resolution genetic monitoring and implications for conservation management of the greater stick-nest rat (*Leporillus conditor*)

Manuscript prepared for submission

Statement of Authorship

Title of Paper	High-resolution genetic monitoring and implications for conservation management of the greater stick-nest rat (*Leporillus conditor*)
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Principal Author

Name of Principal Author (Candidate)	Lauren C White
Contribution to the Paper	Helped design the study, generated and analysed the data, helped interpret the results, drafted the manuscript and produced the figures.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/3/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Vicki A. Thomson
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Contribution to the Paper	Collected samples at Arid Recovery reserve, and helped revise the manuscript
Signature	Date 14/3/2017

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Contribution to the Paper	Collected samples at Mt Gibson sanctuary, St Peter Island and Salutation Island, and helped revise the manuscript		
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Signature		Date	31/3/17

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Contribution to the Paper	Collected samples from the Franklin Islands, helped interpret the results and revised the manuscript.		
Signature		Date	14/3/2017

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Contribution to the Paper	Helped design the study, interpret the results and revise the manuscript.		
Signature		Date	31/3/17

High-resolution genetic monitoring and implications for conservation management of the greater stick-nest rat (*Leporillus conditor*)

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Abstract

Establishment of captive colonies and reintroduced populations are common conservation recovery actions for threatened animals, but often occur once wild populations have become relatively small. Serial founder events, population bottlenecks and genetic drift in small, captive and translocated populations are expected to erode genetic diversity and increase inbreeding. These processes can lead to negative effects, such as inbreeding depression,that will negatively affect the species' long-term sustainability. The greater stick-nest rat (GSNR, *Leporillus conditor*) was formerly distributed through much of southern Australia, but was extirpated from the mainland by the 1930s due to predation by introduced cats and foxes and habitat degradation. The species survived in a single population of ~1,000 individuals on the Franklin Islands off the west coast of South Australia. To alleviate the risk of total extinction, in 1985, a captive breeding and reintroduction program was initiated; this has subsequently resulted in the establishment of five new populations on off-shore islands and within fenced mainland sanctuaries. Despite the success of this program in establishing new populations to reduce the risk of extinction, the species' recent demographic history may pose threats to the long-term survival of these reintroduced populations. We evaluated the genetic diversity in all extant populations of GSNR using the genotype-by-sequencing method, ddRAD-seq to obtain high-resolution measures of genome-wide genetic diversity. Our results show divergence, inbreeding and loss of genetic diversity in all reintroduced populations compared to the Franklin Islands source, and that the translocated populations would benefit from supplementation to increase diversity. Given the divergence of populations due to drift, we suggest that this supplementation consider a targeted approach to sourcing animals from populations with alternative genetic affinities to maximise the genetic diversity in supplemented populations.

Introduction

Founder events, serial population bottlenecks, lack of gene flow and small population sizes are expected to cause increased inbreeding and loss of genetic diversity in populations (Frankham *et al.*, 2010). These processes can lead to inbreeding depression (i.e., increased expression of deleterious traits and decreased fitness) and reduced adaptive capacity, both of which will increase the risk of population extirpation and species extinction (Crnokrak and Roff, 1999; Frankham *et al.*, 1999).

Recovery actions for many threatened animals involve establishment of captive breeding colonies and reintroduction through translocations. There has been increased recognition in recent years that such populations require active management in order to maintain genetic diversity and fulfil the aims of the recovery program (Frankham *et al.*, 2010; Ottewell *et al*., 2014; Weeks *et al*., 2011; Weiser *et al*., 2013). Captive breeding programs often use pedigrees to monitor inbreeding and relatedness, and use this information to select breeding pairs that will maximise the retention of genetic diversity (Ballou and Lacy, 1995). However, such record keeping can be intensive and may be impossible in species that have small body size, which live in groups, have a promiscuous mating system and/or are managed in wild or semi-wild environments (Wang, 2004). In these circumstances, molecular genetic markers can be used to quantify genetic diversity and relatedness within populations (Schwartz *et al.*, 2007). Recent developments in sequencing technology have made the screening of large numbers of loci across the genome practical for most species. This can provide adequate information for genetic management of populations from analyses of small numbers of samples. This information can then guide management actions that will maximise genetic diversity (Deyoung and Honeycutt, 2005), for example, by identifying populations with limited genetic diversity that would benefit from supplementation and identifying appropriate source populations (i.e. ‘genetic rescue’, Hedrick and Fredrickson, 2010).

The greater stick-nest rat (GSNR, *Leporillus conditor*), is a species for which multiple reintroduced and captive populations have been established over the last 30 years. As such, it provides an excellent opportunity to use high-resolution genetic monitoring for guiding targeted management actions.

The greater stick-nest rat is an Australian native, murid rodent that was distributed over much of arid and semi-arid southern Australia (Figure 1, modified from Copley, 1999a). The GSNR mainland populations became extinct in the 1930s, presumably due to predation by introduced European foxes (*Vulpes vulpes*) and cats (*Felis catus*), exacerbated by severe habitat degradation from introduced herbivores (Copley, 1988). The only naturally occurring extant populations of GSNR are on the East and West Franklin Islands in the Nuyts Archipelago, South Australia (Robinson, 1975). Approximately 1,000 GSNR survive on these two islands, which cover approximately 500 ha and are linked at most low tides by a 400 m sand bar (Copley, 1999a).

Given the precarious nature of the species' survival, a captive breeding and reintroduction program was initiated in 1985 (Copley, 1999b). The program involved the founding of a captive colony that was then used to found several reintroduced populations (Copley, 1999b). Later reintroductions were founded by animals from the previously-established reintroduction sites and the Franklin Islands (Moseby *et al*., 2011; Moseby and Bice, 2004; Page et al., 2011). There are now five reintroduced populations of GSNR (Figure 1): three on offshore islands and two inside fenced mainland reserves, increasing the total population size of the species four-fold (Woinarski and Burbidge, 2016). These improvements have led to the species' IUCN conservation status being downgraded twice: from endangered to vulnerable in 1999 and from vulnerable to 'near threatened' in 2008 (Woinarski and Burbidge, 2016).

Despite these achievements, the adaptive capacity and genetic diversity within reintroduced populations is of concern, as genetic diversity has not been measured since the early stages of the conservation program (Barclay *et al*., 2006; Copley, 1999b). Additionally, after 12 years in captivity, the original captive colony of GSNR was found to express a high incidence of cataracts, which can ultimately lead to blindness (Copley, 1999b). Further investigation found that the disorder was present in all wild populations, but at much lower frequencies (Copley, 1999b). This suggests that the condition has a genetic component and unavoidable inbreeding in the small captive population led to its increased expression. Given the known predisposition to a putative genetic disorder and the serial founder events experienced by the GSNR, it is important that this species continues to be managed so that genetic diversity is maximised.

In our study, we used the genotyping-by-sequencing method, ddRAD-seq (Poland *et al.*, 2012) to genotype thousands of single nucleotide polymorphism (SNP) markers in individuals from all seven extant populations of GSNRs. Using this large, high-resolution dataset, we specifically aimed to i) determine the baseline genetic diversity in the source populations (East and West Franklin Islands), and use this to measure the relative levels of diversity in all reintroduced populations, and ii) make recommendations about the need for 'genetic rescue' in the established reintroduced populations, and the most appropriate source or sources for any future supplementation of these populations.

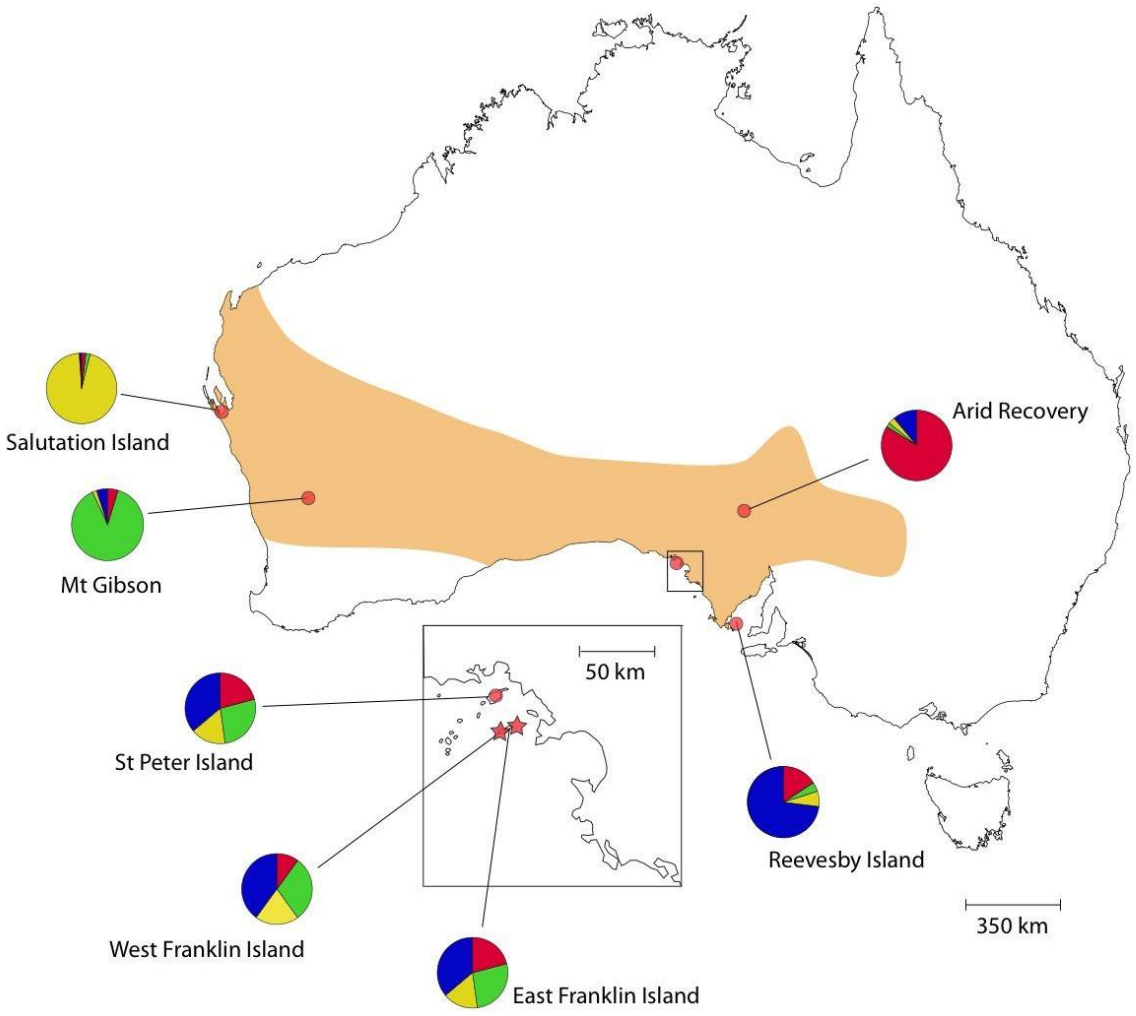

Figure 1. Former (orange) and current (red) distribution of the greater stick-nest rat. Red stars represent the only remaining natural populations of the GSNR at the Franklin Islands and the red circles represent reintroduction sites. Pie charts show the inferred ancestry proportions from sNMF analysis (see below). The Monarto captive breeding colony is not shown as it was discontinued in 2004.

Reintroduction History and Background

The reintroduction history of the GSNR is summarised in Table 1. The conservation program for GSNR began when 29 individuals were transferred from the Franklin Islands to

Monarto Zoological Park, South Australia between 1985 and 1998 to found a captive colony, which was used subsequently as a source for several reintroductions (Table 1; Copley, 1999b). Between 1990 and 1991, 101 individuals from Monarto were reintroduced to Reevesby Island (344 ha) in the Spencer Gulf of South Australia (Pedler and Copley, 1993). Also in 1990, 40 Monarto individuals were released at Salutation Island (163 ha) in Shark Bay, Western Australia (Copley, 1999b; Morris, 2000). Another 153 individuals from Monarto were released at St Peter Island (4,028 ha) in Nuyts Archipelago between 1993 and 1998 (Copley, 1999b). Between 1998 and 2004, 98 individuals from Reevesby Island and eight from the Monarto colony were released at Arid Recovery Reserve (6,000 ha) near Roxby Downs in South Australia (Moseby *et al*., 2011; Moseby and Bice, 2004). Finally, in 2011, 39 individuals from the Franklin Islands were used to found the most recent GSNR reintroduction at a 7,800 ha fenced site at Mt Gibson Wildlife Sanctuary in central-south Western Australia (Page *et al*., 2011). The Mt Gibson population was supplemented with ten animals from a captive colony at Alice Springs Desert Park (which was founded from Arid Recovery animals) in 2014 (L. Ruykys, *pers. comm*, B. Pascoe, *pers. comm*). Five other reintroductions have been attempted (Venus Bay Peninsula, Yookamurra Sanctuary, Scotia Sanctuary, Heirisson Prong, and Faure Island). The outcome of the Scotia reintroduction is uncertain; the others have failed due to high levels of predation, small founding group size and other unknown reasons (Copley, 1999b; Page *et al*., 2011, J. Kanowski, *pers. comm.*).

Since the conservation program began, the total population of the species has increased four-fold. While the Franklin Islands population has remained steady at approximately 1,000 individuals, there are now around 1,000 individuals on Reevesby Island, 200 on Salutation island, 1,000 on St Peter Island, 600 at Arid Recovery and <100 at Mt Gibson (Woinarski and Burbidge, 2016, Page *pers comm*, Moseby *pers comm*). These population sizes are estimates as monitoring has not been undertaken recently. The captive breeding program at Monarto

Zoological Park ceased in 2004 due to the de-prioritisation of the colony after the establishment of three reintroduced populations (Copley, *pers comm*).

Table 1. Summary of reintroduction history of the greater stick-nest rat. Populations are ordered by date of reintroduction. Populations denoted with an asterisk are captive colonies of GSNRs.

Population	Island/ Reserve Area (ha)	Year Sampled (this study)	Population Size (at time of sampling)	Number of Founders	Founder Source	Translocation Year
West Franklin	247	1994	~500	-	-	-
East Franklin	225	1994	~500	-	-	-
Monarto*	-	1999	-	29	Franklin Islands	1985-1998
Reevesby Island	344	1999	~1000	101	Monarto	1990-1991
Salutation Island	163	2016	~200	40	Monarto	1990
St Peter Island	4028	2016	~1000	153	Monarto	1993-1998
Arid Recovery	6000	2016	~600	98, 8	Reevesby Island, Monarto	1998-1999
Mt Gibson	7800	2016	<100	39, 10	Franklin Islands, Alice Springs Desert Park	2011-2014
Alice Springs Desert Park*	-	Not Sampled	-	6	Arid Recovery	2009

Methods

Sample Collection

We sampled animals from all seven extant populations of the GSNR (East and West Franklin Island, Reevesby Island, St Peter Island, Salutation Island, Arid Recovery and Mt

Gibson) and from the former captive colony at Monarto Zoological Park. Tissue samples were obtained from museum collections, during routine monitoring programs, or through targeted trapping opportunities. Samples from the Franklin Islands, Reevesby Island and Monarto captive colony animals were respectively taken during monitoring on the Franklin Islands in 1994, and during trapping for the Arid Recovery reintroduction in 1998-9 on Reevesby Island and Monarto. These samples were stored frozen at the Australian Biological Tissue Collection (ABTC, South Australian Museum) and subsampled for this study. Animals from all other populations were trapped in 2016 using Elliot traps or Sheffield cage traps baited with peanut butter and rolled oats, or fresh fruit/vegetables. Ear or tail tissue samples were taken using an ear punch, small sharp scissors or sterile scalpel blade, and stored in individual vials of ethanol. Samples were stored frozen until extraction. Ethics approval was sought for all trapping conducted as part of this study. Permit numbers are given in SI Table 1.

Samples from Arid Recovery, Reevesby Island and Monarto were collected and sequenced as part of a previous study by White *et al.* (Chapter 5). Reevesby and Monarto samples represent the founding animals of the Arid Recovery population.

DNA Extraction

DNA extraction was performed using a salting out method. Tissue samples stored in ethanol were air dried for 45 minutes prior to digestion. Samples were digested overnight at 55°C in 300 µL of lysis buffer (10mM Tris, 0.1M EDTA pH8 and 2% SDS), 60 µg of proteinase K, and 0.08 M dithiothreitol (DTT). Digested samples were incubated at 37°C with 1 µL of RNase A (10 mg/ml; Thermo Scientific) for 30 minutes. After digestion, 100 µL of 7.5 M ammonium acetate was added, the mixture was vortexed and left on ice for an hour. The samples were then centrifuged at 13,000 rpm for 5 minutes and the pellet was discarded.

The supernatant was mixed with 300 µl of 100% isopropanol and 0.5 µl of glycogen (20mg/ml, Sigma-Aldrich). The mixture was mixed gently by inversion and then spun at 15,000 rpm for 10 minutes. The supernatant was discarded and the pellet washed in 300 µL of 70% ethanol and then air dried for 30 minutes. The DNA pellet was re-suspended at 65°C for an hour in 40 µL of TLE buffer (10 mM Tris, 0.1 M EDTA, pH 8). DNA extracts were quantified using the Quantus Fluorometer system (Promega) as per the manufacturer's instructions.

ddRAD-seq Library Preparation

We generated double-digest restriction associated (ddRAD) libraries consisting of 95 samples and a library blank following the protocol of Poland *et al*. (2012) with some modifications. Digestion and ligation reactions were performed in 96-well plates. We digested 300 ng of each DNA extract at 37°C for 2 hours using 8 U of the restriction endonucleases *PstI* and *HpaII* in 20 µL of 1x CutSmart Buffer and H_2O (New England Biosciences [NEB]). *PstI* is a rare cutting enzyme with a six-base recognition site (CTGCAG) and *HpaII* is a more common cutting enzyme with a four-base recognition site (CCGG).

We then ligated uniquely barcoded adapters (see SI methods and SI Table 2) to the sticky ends of the digested fragments. Ligation reactions were performed in 40 µL volumes consisting of 20 µl of digested DNA, 200 U of T4 ligase, 0.1 pmol of forward (rare) and 15 pmol of reverse (common) adapters (SI Figure 1), 1x T4 Buffer and H_2O. The mixture was left at room temperature for 2 hours, and then heat killed at 65°C for 20 minutes. We pooled the ligation products into 12 libraries of 8 samples each. Pooled libraries were purified using the QIAqiuck PCR purification kit (Qiagen) and eluted in 120 µL of EB buffer (Qiagen).

PCR reactions to add the full-length Illumina adapters (Poland *et al*., 2012) were performed in 8 replicates per library in 30 µL volumes containing 10 µL of purified library,

1x Hot Start Taq Master Mix (NEB), 0.66 µM each of the forward and reverse primers (SI Figure 1) and H_2O. The PCR conditions were as follows: 95° C for 30 seconds, 16 cycles of 95° C for 30 seconds, 65° C for 20 seconds, and 68° C for 30 seconds, followed by 68° C for 5 minutes, and 25° C for 1 minute. The eight replicates per library were re-pooled and purified as above, eluting in 30 µL of EB buffer (Qiagen). We employed a two-step double-SPRI protocol (Lennon *et al.*, 2010) to select for fragments between 100 and 300 bp using a homemade SPRI bead mix (Rohland and Reich, 2012). Libraries were then quantified using Tapestation 2200 (Agilent) and pooled at equi-molar concentrations. Pooled libraries were sequenced in 1x75 bp (single-end) high output reactions on the Illumina Next-seq at the Australian Genome Research Facility, Adelaide.

S*equence Processing*

We used STACKS v1.35 (Catchen *et al*., 2013, 2011) to process the ddRAD-seq data employing parameters recommended by Mastretta-Yanes *et al.* (2015) to minimise errors and maximise SNP recovery. Raw sequencing reads were de-multiplexed, truncated to 65 bp, and filtered for overall quality based on the presence of barcodes using the *process_radtags* module. Samples with fewer than 500,000 reads were excluded from downstream analysis. RAD loci were identified for each sample using the *ustacks* module, requiring a minimum stack read depth of three (m=3) and a maximum of two nucleotide mismatches (M=2) between stacks at a locus. Loci with more than three stacks (mls=3) and more reads than two standard deviations above the mean were filtered as they may map to multiple points on the genome. A 'deleveraging algorithm' was used to try to resolve over-merged loci. A catalogue of consensus loci among individuals was constructed with the *cstacks* module using the *ustacks* output files. Loci were recognized as homologous across individuals if there were two or fewer mismatches between the consensus sequences (*n*=2). Alleles were identified in each

individual against this catalogue using the module *sstacks*. The module *populations* was used to remove potential homologs by filtering loci with heterozygosity > 0.7 and the resulting SNP datasets were output to a PLINK format file (i.e. ped and map files). Finally, the program PLINK (Purcell *et al*., 2007) was used to filter loci with more than 25% missing data and minor allele frequencies of < 0.05.

Quality Control

Raw sequences from blank control samples were also run through the STACKS pipeline, matching the *ustacks* output to the consensus catalogue. Our aim was to remove any potentially erroneous loci in our dataset that were also present in the library blank samples. However, upon inspection, none of the loci found in the blank controls were present in the final datasets, having been removed by the filtering steps.

To allow the estimation of error rates, ten samples, representing individuals from four of eight populations, were sequenced twice in separate libraries. To control for sequencing depth, replicate reads were subsampled to 1 million, 750,000, and 500,000 reads. All subsampled replicates were run through the STACKS pipeline as above, matching the *ustacks* output to the previously-constructed consensus catalogue. Allelic error rate was then estimated by counting mismatching alleles at loci for which both replicates had been sequenced.

Genetic Diversity and Inbreeding

We calculated expected and observed heterozygosity (H_E , H_O), and allelic richness corrected for sample size (A_R) for each population using the R package *hierfstat* (Goudet, 2005). We treated East and West Franklin separately based on F_{ST} values that indicated that the two populations were significantly differentiated (see below). Individual inbreeding

coefficients (*F*) were calculated in PLINK (Purcell *et al.*, 2007). We tested for significant differences in *F* between the five reintroduced population and the two source populations (East and West Franklin) using a Wilcoxon rank sum test, corrected for multiple testing.

Finally, Wang's pairwise relatedness coefficient (*PR*, Wang, 2002) was estimated for all pairs of individuals within and between all populations using the R package *Related* (Pew *et al.*, 2015). *PR* measures the degree of genetic similarity between two individuals relative to the average genetic similarity in the total sample (Hardy, 2003). Consequently, negative values may be obtained if two individuals are less related than the average in the reference.

Population Differentiation

We visualised the variation in our datasets by performing a principal components analysis (PCA) in *adegenet v2.0.1* (Jombart, 2008) . PCA is a statistical method for exploring datasets that have a large number of measurements; it works by reducing the variation in the dataset to a few principal components, which can then be projected onto a graph (Reich *et al.*, 2008). Genetic distance between populations was measured as pairwise F_{ST} in Arlequin v3.5 (Excoffier and Lischer, 2010) using the underlying pairwise distance matrix and 10,000 permutations. Significance values were corrected for multiple tests using the Bonferonni correction (Rice, 1989).

We then used the program sNMF v1.2 to examine the proportional ancestry in all populations of GSNR (Frichot *et al.*, 2014). Similar to the widely-used program STRUCTURE (Pritchard *et al.*, 2000), sNMF estimates the proportion of each individual's genome that originated from a specified number of gene pools (K). Unlike STRUCTURE, sNMF is capable of efficiently analysing large SNP datasets and is more robust to many of the demographic assumptions of Hardy-Weinberg and linkage equilibrium (Frichot *et al.*, 2014). We calculated ancestry proportions in our dataset by running ten replicates of K 1-20

with default parameters and chose the best-supported K as the one with the lowest cross-entropy criterion (CEC), as calculated in sMNF.

We tested for loci under putative selection using the Bayesian FST-outlier method implemented in Bayescan v2.01 using the default settings (Foll and Gaggiotti, 2008). Bayescan estimates the probability that each locus is subject to selection by teasing apart population-specific and locus-specific components of F-coefficients using a logistic regression. Using a reversible jump Markov chain Monte Carlo (MCMC) algorithm, the posterior probability of a locus being under selection is assessed by testing whether the locus-specific component is necessary to explain the observed pattern of diversity, which infers a departure from neutrality. A threshold value to detect selection was set using a conservative maximum false discovery rate (the expected proportion of false positives) of 0.01.

Results

Sequencing

A total of 146 GSNR individuals from seven extant populations and the former captive colony at Monarto were successfully sequenced. Samples had an average 4,323,612 reads that passed quality filtering. After processing and filtering, a final dataset of 8,723 SNPs was generated, with an average of 9.86 % of loci missing per individual (SI Table 3). The estimated average allelic error rates, calculated between pairs of replicates subsampled to varying depths, is shown in Table 2. The error rate did not differ with sequencing depth (mean = 2.5%), indicating that our cut-off of 500,000 reads per sample was appropriate.

Table 2. Average allelic error rates calculated for paired replicates, subsampled to different depths for the single nucleotide polymorphisms identified using ddRADseq.

	Number of Reads in Subsampled Replicate A			
		1 Million	**750k**	**500k**
Number of Reads Subsampled in Replicate B	**1 Million**	0.026	0.024	0.025
	750k	0.025	0.024	0.024
	500k	0.026	0.026	0.025

Genetic Diversity and Inbreeding

Allelic richness, and observed and expected heterozygosity, was highest in the Franklin Islands source populations and lowest in the introduced Mt Gibson and Salutation Island populations (Table 3). Mt Gibson, which is still in the early phases of establishment, was the only population in which the observed heterozygosity is higher than the expected heterozygosity.

Table 3. Measures of genetic diversity in wild, captive and introduced populations of greater stick-nest rat. Number of sampled individuals (n), allelic richness corrected for sample size (A_R), and expected and observed heterozygosity (H_E, H_O)

	n	**A_R**	**H_E**	**H_O**
West Franklin	7	1.34	0.35	0.32
East Franklin	8	1.34	0.34	0.31
Monarto	6	1.32	0.33	0.30
Reevesby Island	72	1.33	0.33	0.31
Salutation Island	19	1.28	0.28	0.27
St Peter Island	9	1.32	0.32	0.30
Arid Recovery	17	1.30	0.30	0.30
Mount Gibson	8	1.27	0.27	0.30
Max. difference		0.07	0.08	0.05

All reintroduced populations had a higher average individual inbreeding than the two source populations (Figure 2), but the only populations that were significantly different from the Franklin Islands were Salutation Island and Arid Recovery. As expected, *PR* was highest within populations (SI Table 4, Figure 3). Mt Gibson had the highest within-population *PR*, and East and West Franklin had the lowest (Figure 4). The lowest average between-population *PR* was between comparisons of Salutation Island and all other populations (SI Table 3).

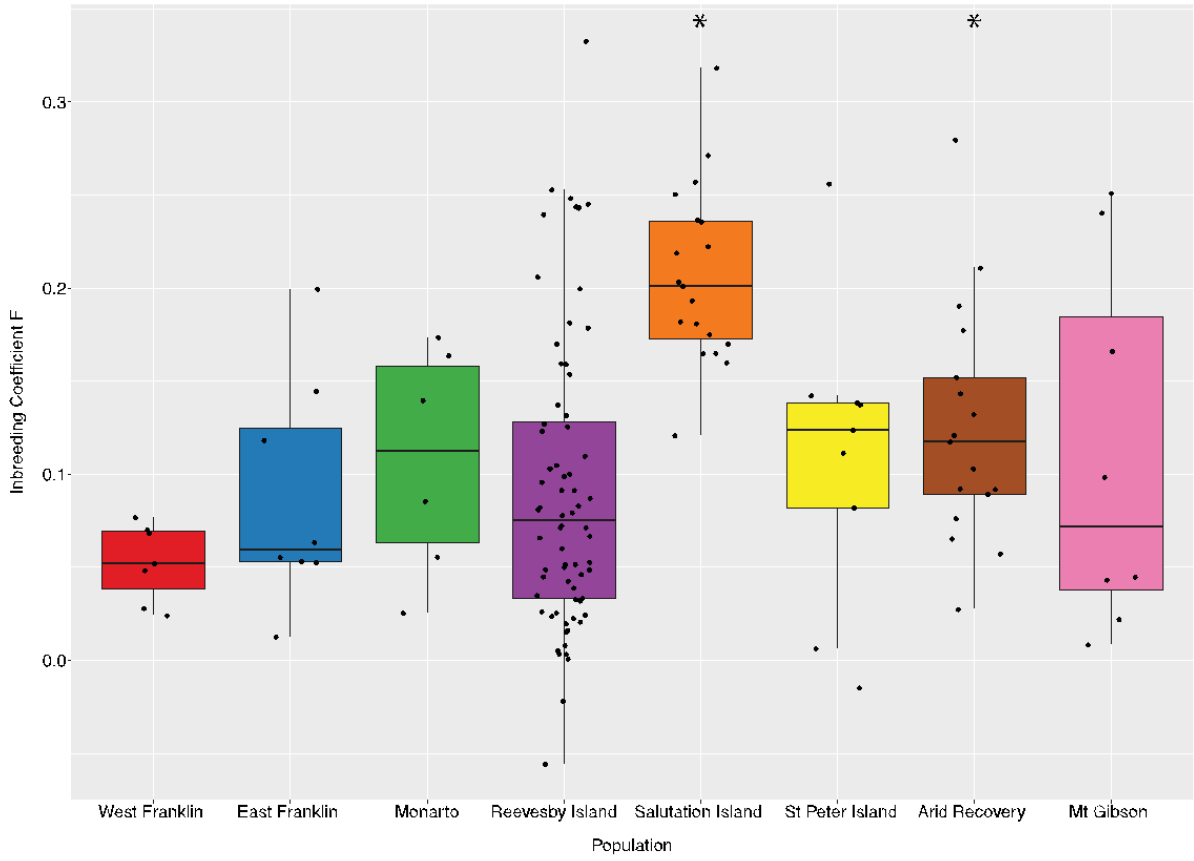

Figure 2. Individual inbreeding coefficients per population for wild, captive and introduced populations of greater stick-nest rat. Dots represent individual values. Middle horizontal lines represent the median, the boxes are bound by the 25th and 75th quartile and vertical lines show the minimum and maximum range of values excluding outliers. Reintroduced populations with average inbreeding coefficients that were significantly different from both the Franklin

Islands are denoted with an asterisk. Populations are ordered by the date of first translocation with the two source populations (East and West Franklin) first.

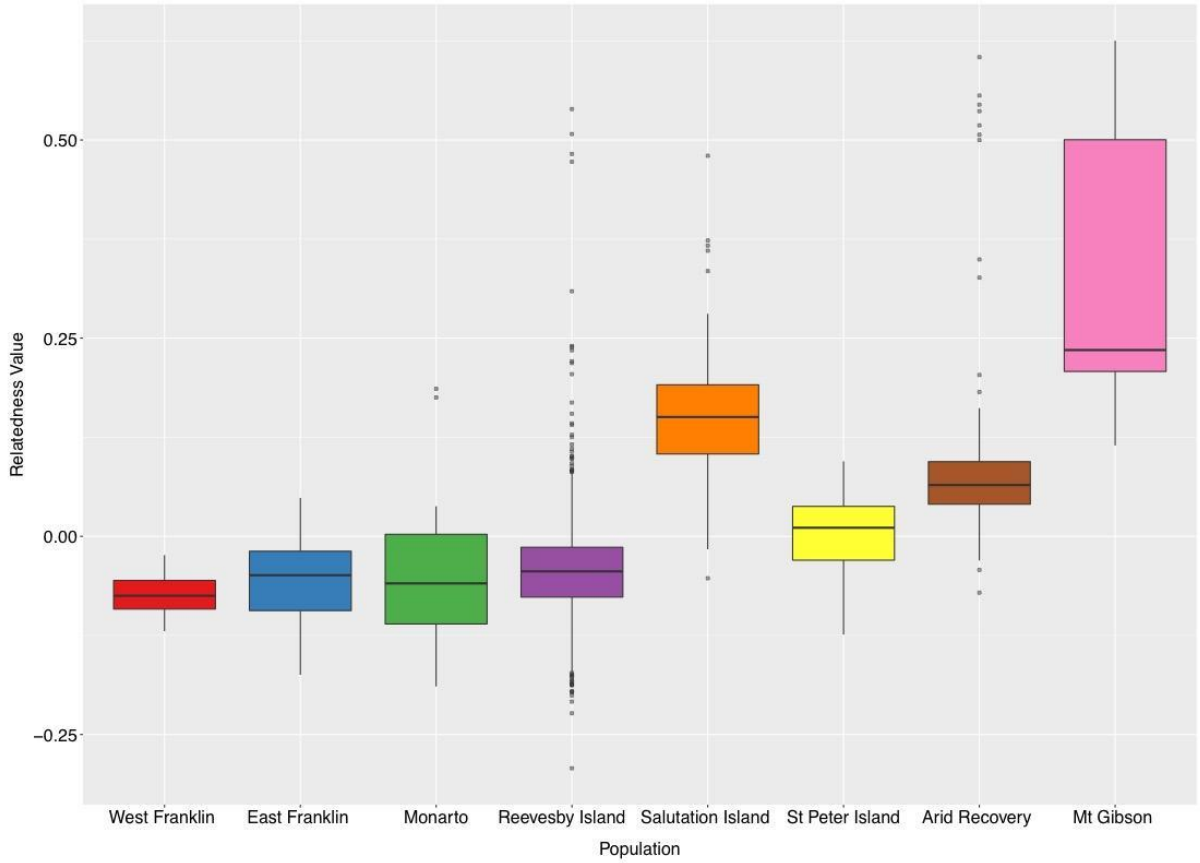

Figure 3. Pairwise relatedness of individuals within each population of wild, captive and introduced populations of greater stick-nest rat. Middle horizontal lines represent median values, the boxes are bound by the 25th and 75th percentiles and the vertical lines represent the minimum and maximum values excluding outliers. Outliers are represented by dots. Populations are ordered with the two source populations (East and West Franklin) to the left, then by date of first translocation to the right.

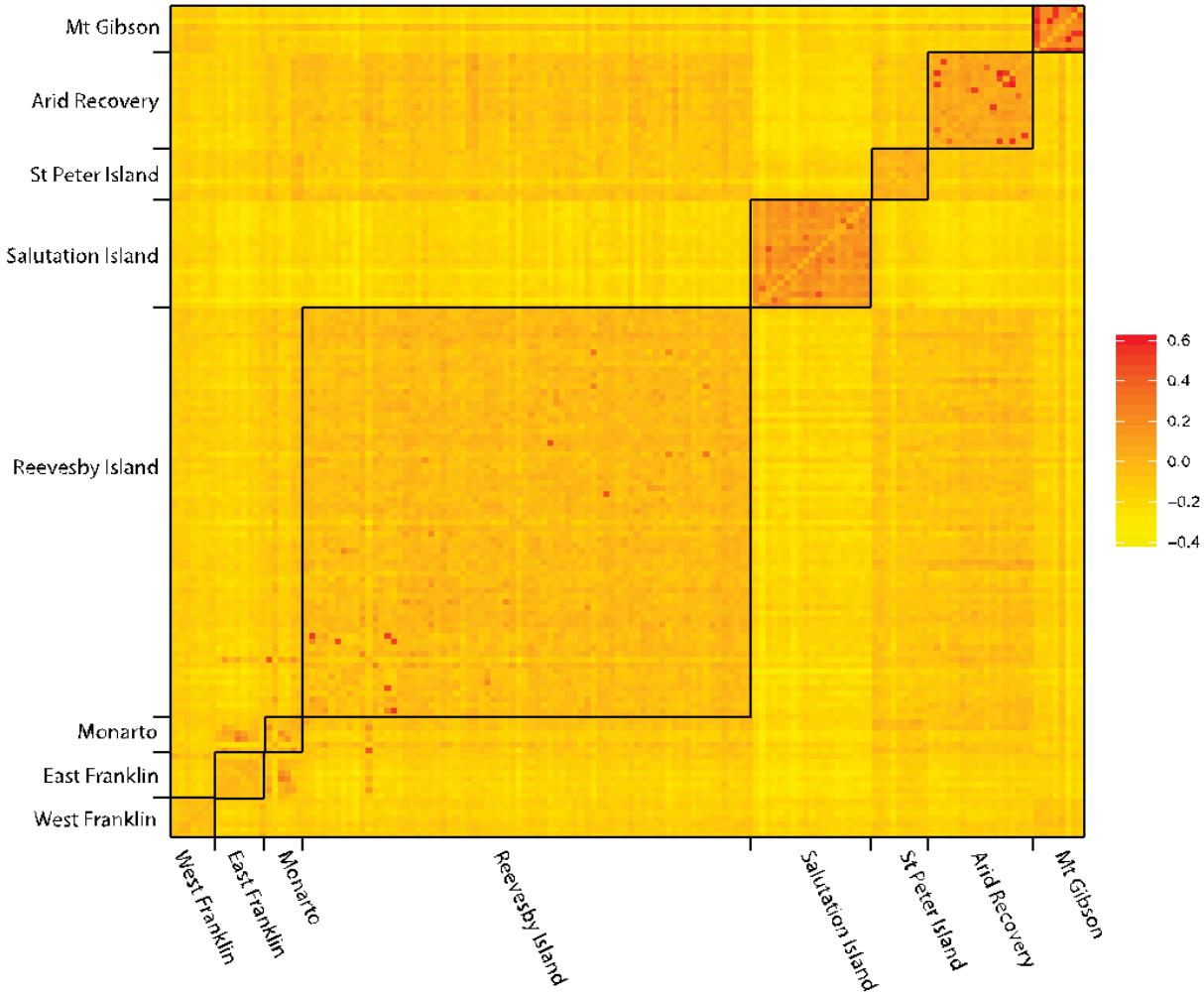

Figure 4. Heat map of pairwise relatedness between all sampled individuals from wild, captive and reintroduced populations of greater stick-nest rat. Within population comparisons are bounded by black squares.

Population Differentiation

Most populations are identifiable as clusters on the PCA graphs of principle components 1-3, except the Monarto group, which largely overlaps with the Franklin Islands, Reevesby Island and St Peter Island populations (Figure 5). The Mt Gibson, Salutation Island and Arid Recovery populations appear the most diverged from the Franklin Island

populations, and the two Franklin Island populations are slightly separated. However, the total amount of variation explained in each of these principle components is small (2.39-5.01%).

In agreement with our PCA, pairwise F_{ST} was low overall (Table 4); being highest between the Mt Gibson and Salutation Island populations (0.206), and lowest between the East Franklin Island and Monarto populations (0.00). After correction for multiple tests, most pairwise F_{ST} measures were significantly different from zero, except for between Monarto and the Franklin Islands, Reevesby Island and St Peter Island. Pairwise difference between the two Franklin Islands was relatively low (0.037), but significantly different from zero.

Figure 5. Relationships among wild, captive and introduced populations of greater stick-nest rat based on Principle Coordinate Analysis for components 1, 2 and 3. Each dot represents an individual coloured by population. Ellipses represent the centre and 95% confidence interval of the points in each population.

Table 4. Pairwise population F_{ST} values between the wild, captive and introduced populations of greater stick-nest rat. Values that are significantly different from zero are in bold.

	West Franklin	East Franklin	Monarto	Reevesby Island	Salutation Island	St Peter Island	Arid Recovery
East Franklin	**0.037**						
Monarto	0.015	0.000					
Reevesby Island	**0.042**	**0.056**	0.016				
Salutation Island	**0.117**	**0.138**	**0.110**	**0.118**			
St Peter Island	**0.050**	**0.052**	0.006	**0.039**	**0.130**		
Arid Recovery	**0.085**	**0.091**	**0.045**	**0.048**	**0.168**	**0.080**	
Mount Gibson	**0.110**	**0.144**	**0.130**	**0.139**	**0.206**	**0.161**	**0.176**

The sNMF analysis inferred that four was the most likely number of gene pools (K=4), based on the CEC (SI Figure 2). We interpret the ancestry estimates from sNMF, visualised in Figure 1 and 6, as showing the divergence (due to drift, inbreeding and/or selection) of four reintroduced populations from the source populations. The percentage of ancestry in individuals from four reintroduced populations (Mt Gibson, Reevesby Island, Arid Recovery and Salutation Island) are dominated by one 'gene pool,' while all four 'gene pools' are present in roughly equal proportions in the Franklin Islands, Monarto and St Peter Island populations.

Bayescan identified 41 loci under putative selection (SI Figure 3), which represents 0.5% of all SNPs genotyped. These 41 loci had F_{ST} values of > 0.25 compared to an average across all loci of 0.09.

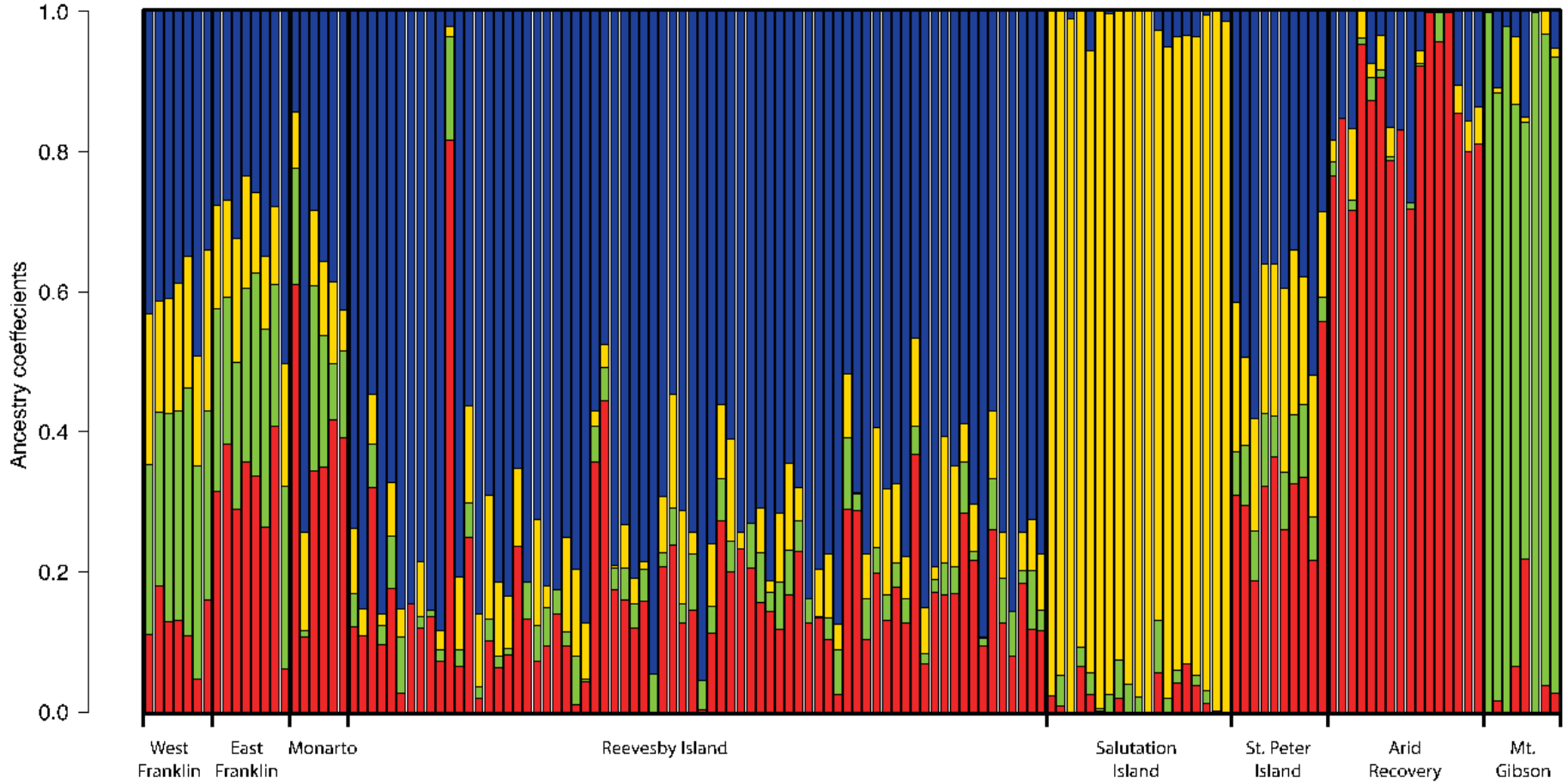

Figure 6: Genetic ancestry in individuals from wild, captive and introduced populations of greater stick-nest rat estimated using sNMF. Each vertical bar represents an individual

Discussion

As expected from genetic theory, our analysis of genetic diversity in GSNR reveals a reduction in genetic diversity, increase in inbreeding, and some genetic differentiation, among reintroduced populations of the GSNR compared to the source populations on the Franklin Islands. Our high-resolution analysis using ddRAD SNP data provides a sound basis for having a strategic approach to targeted sourcing of animals for supplementation of populations so as to maximise genetic diversity and overcome differentiation due to drift. Our study demonstrates the value of genetic analysis in management of wild and translocated populations and planning of recovery actions.

Genetic Diversity, Inbreeding and Recommended Supplementation

As expected, genetic diversity (measured by A_R, H_O and H_E) was lower and inbreeding higher in the reintroduced populations of GSNR compared to the original wild source population at the Franklin Islands. It is not known whether the loss of genetic diversity and increased inbreeding that we observed corresponds with possible changes, if any, in the fitness of individuals within populations. However, three reintroduced populations had higher inbreeding than the Monarto individuals, which were sampled in 1999—after the putative-genetic eye disorder was characterised (Copley, 1999b). A genetic link to this disorder has not been confirmed, but a reassessment of the frequency of cataracts in the reintroduced populations may be warranted. Future studies on the possible genetic basis of the disease (or otherwise) would be greatly assisted by the development of a reference genome for GSNR (Allendorf *et al.*, 2010).

While it is unclear how the small amount of genetic diversity loss and inbreeding that we observed will impact the health and long-term sustainability of GSNR populations,

maintaining genetic diversity at > 95% of source population levels is a conservative measure often adopted in management programs (Lacy, 1987; Weeks *et al*., 2011) and it is on this basis that we make recommendations on the need for additional translocations.

The Arid Recovery and Salutation Island populations are the most inbred and have lost more than 5% of the Franklin Islands diversity across most measures. These populations also had some of the highest within-population pairwise relatedness. We suggest that these two populations would benefit the most from supplementation to maintain genetic diversity at or near source population levels. In contrast, a recent study focusing on Arid Recovery reserve did not recommend additional translocations of GSNR to that population (White *et al.* Chapter 5). While White *et al.* (Chapter 5) measured the change in genetic diversity at Arid Recovery compared to the founding individuals of that population, our study here measured change in genetic diversity compared to the original source populations at the Franklin Islands. Thus, the dataset presented here could detect the true impact of serial founder effects that was not discernible in the previous study. This highlights the benefits of sampling widely from all available populations in genetic monitoring programs of managed species.

While the Mt Gibson population has also lost > 5% of genetic diversity compared to the East and West Franklin islands, this population shows an excess of heterozygosity and the associated individual inbreeding (despite a high variance) was not significantly different from the source populations. Excess heterozygosity is common in populations that have undergone a recent bottleneck (Cornuet and Luikart, 1996) and it is possible that not enough generations have passed since release for inbreeding to accumulate to the levels seen in Arid Recovery or Salutation Island. On the other hand, average pairwise relatedness was high, reflecting the small population size (< 100) since release in 2011. While individual inbreeding coefficients describe how related an individual's parents were, pairwise relatedness describes how inbred the potential offspring of two individuals is likely to be (Hedrick and Lacy, 2015). We

therefore expect inbreeding to increase at Mt Gibson in subsequent generations, and suggest it would benefit from further supplementation.

We note that the samples from the Franklin Islands and from Reevesby Island were taken > 17 years ago and may not reflect the current genetic diversity in those populations. The Franklin Islands population was stable during initial monitoring in the 1980-90s and, although it has not been monitored since, we assume that the stable state has continued. We therefore do not expect the genetic diversity of that population to have changed significantly since sampling in 1994. However, monitoring of the Reevesby Island population showed a boom and bust cycle after release (Pedler and Copley, 1993), which would be expected to lead to a reduction in genetic diversity and increase in inbreeding. More sampling of both these populations is needed to test these predictions.

Population Differentiation

Genetic differentiation between the two Franklin Islands, which make up the remaining wild population of GSNR, was low, but significantly different from zero. This suggests that the daily flooding of the sandbar between the islands represents an incomplete barrier to gene flow. The decision to source animals for establishment of the captive colony from both islands in order to maximise genetic diversity (Copley, 1999b) would be expected to lead to low differentiation between the Monarto population and the source. Our data is consistent with this expectation, with very low, non-significant F_{ST} values between Monarto and the Franklin Islands.

While serial sourcing of animals for translocation might be expected to maintain similar levels of genetic relatedness, multiple bottlenecks may lead to skewed patterns of differentiation due to drift. Analysis of pairwise F_{ST} shows significant divergence of all reintroduced populations from the two source populations. Analysis of relationships among

populations using PCA and sNMF, indicates that this divergence from the source populations has occurred in different 'directions', and is greatest in the Salutation Island, Mt Gibson and Arid Recovery populations.

The level of divergence between Mt Gibson and the source populations is surprising given that this is a recent translocation. This may be due to small sample size or change in genetic make-up in the Franklin Island population between sampling in 1994 and translocations of individuals to Mt Gibson in 2011. Resampling the Franklin Island and Mt Gibson populations is necessary to test this hypothesis. However, the population size at Mount Gibson is likely less than 100 individuals and possibly as low as 20 (L. Ruykys *pers. comm.*). Such a small census size would result in a very small effective population size, in which high amounts of drift would be possible over a small number of generations as observed. If confirmed, this pattern shows the importance of initial population growth after release in maintaining genetic diversity in reintroduced populations.

The observed patterns of divergence in the GSNR populations could be due to selection in different habitats. For example, the Arid Recovery population experienced high mortality after release due to heat stress in summer, which would have reduced founder sizes and may have acted as a selective pressure on that population (Moseby, *pers comm*). There are also significant ecological differences between many of the reintroduction sites and the Franklin Islands: the Arid Recovery reserve is an area of chenopod swales (*Atriplex* spp.) and wattle/hopbush sand dunes (*Acacia* spp./*Dodonaea* spp; Moseby *et al*., 2011), which contrasts with both the rocky, shrub-dominated habitat of the island environments (Copley, 1999a), and the area of mixed wood- and shrub-land at Mt Gibson sanctuary (Page *et al.*, 2011). St Peter Island is also the closest geographically and ecologically to the Franklin Islands, and it is interesting that the sNMF analysis shows that this population has maintained a similar level of genetic admixture to the Franklin Islands. However, the observed patterns of

divergence may also be an artefact of a bottleneck at establishment in many of the reintroduced populations.

We tested for selection in the genotyped loci using Bayescan, which identified 41 loci that had higher deviation than expected and represent loci potentially under selection. F_{ST} outliers can also be caused by the stochastic effects at the wave-edge of an expanding population (Hofer *et al.*, 2009; Klopfstein *et al*., 2006). Given the recent expansion of the reintroduced populations, and the likely small effective population size that would limit natural selection (Frankham *et al.*, 2010), the scenario of genetic drift may be more likely for extant GSNR populations. We emphasize that the outlier loci identified here should be treated as candidates of selection which require further, detailed investigation (Bierne *et al*., 2013).

Recommendations on Sourcing for Future Translocations and Reintroductions

As expected, the original Franklin Islands populations had the highest genetic diversity, lowest inbreeding and lowest within-population pairwise relatedness, and therefore should be considered the best source for any future reintroductions. However, given these samples were from over 20 years ago and given the high differentiation of the most recent translocation from this population, we recommend re-analysing the genetic diversity of this wild population prior to any further use as a source of animals.

The pairwise relatedness analysis suggests that the mixing of diverged populations (for example Salutation Island with another reintroduced population) could be considered as a strategy to maximise diversity and minimise inbreeding in the reintroduced populations and during the establishment of future reintroductions. The strategy of admixing slightly diverged populations was examined in a study on reintroduced Alpine Ibex, which, like GSNR, are all descended from a single ancestral population (Biebach and Keller, 2012). The study found that admixture between populations with even a small amount of divergence had a greater,

positive effect on genetic diversity than sourcing from a single ancestral population. Similarly, assisted translocations between diverged populations of mountain pygmy possums in Victoria, Australia dramatically increased genetic diversity and reproductive success in a failing population (Weeks *et al.*, 2015).

The 'admixture strategy' relies on animals from both populations contributing equally to the descendent population; however, this cannot be guaranteed in free-living groups. Despite these risks, the admixture strategy may still be preferred in certain situations, for example when the reintroduction site is geographically closer to two reintroduced populations than a single natural source. In this case, using animals from the reintroduced populations may be more cost-effective and would minimise the stress placed on the founders during transport, potentially having a positive impact on reintroduction success (Dickens *et al.*, 2010; Teixeira *et al.*, 2007). Given the divergence and alternate genetic affinities of the Arid Recovery, Salutation Island, Reevesby Island and Mt Gibson populations, targeted sourcing of animals for reciprocal translocations and admixture between these populations could be considered as a tool for maximising genetic diversity in GSNRs.

Conclusion

Our high-resolution dataset has provided the first species-wide assessment of genetic diversity in GSNR and demonstrated that while genetic diversity shows some reduction in translocated populations as might be expected based on genetic theory, the divergence of populations through genetic drift was unexpected. This has enabled recommendations for a strategic approach to targeted sourcing of animals to supplement existing populations and maintain genetic diversity, which is important given planned supplementation of the Mt

Gibson population and the planned reintroduction of GSNR to Dirk Hartog Island, Western Australia in the near future. Furthermore, we have demonstrated the benefits of genetic monitoring for successful management of endangered species and in planning translocation events, and recommend it as a key tool in whole of species management strategies for mammals subject to active recovery programs.

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Supplementary Information

Supplementary Methods: Design and Preparations of Barcoded Adapters

Both the barcoded forward primer and the common reverse primer (a Y-adapter) were designed as per Poland *et al*. (2012; see SI Figure 1). A set of 96 barcodes were designed using the barcode-generator python script (https://github.com/audy/barcode-generator) to range in size from 5-9 bp in length with a Levenstein distance of at least 3 to allow samples to be distinguished from one another even with one sequencing error in each barcode (see SI methods). The single stranded oligonucleotides of each barcode adapter and the common adapter were resuspended to 100 µM in 1x Elution Buffer (EB; 10mM Tris-Cl, pH=8.0). To make a plate of working aliquots for the double stranded adapters, we added 10 µl of each single stranded oligo (at 100 µM) to 10 µl of 10x Adapter Buffer (AB; 500mM NaCl, 100mM Tris-Cl) and 70 µl of H_2O. This mixture was then heated to 95° C for 2 minutes, and cooled at 1° C per minute until 30° C was reached, and then held at 4° C for 5 minutes. The barcoded adapters were then diluted 3:10 with AB and quantified using Quant-iT Picogreen dsDNA dye (Invitrogen) on a Quantus fluorometer (Promega Corporation). Each barcoded adapter was normalised to 1.6 ng/µl (=0.1µM). A plate containing a combination of the forward barcoded adapter and common reverse adapter was then prepared by adding 20 µl of the barcoded adapter (at 0.1 µM) to 30 µl of the common reverse adapter (at 10 µM) and 50 µl of 1x AB.

SI Figure 1. Configuration of barcoded adapters and full length Illumina sequencing adapters.

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SI Table 1: Permit numbers for all trapping conducted as part of this study.

Location	Permit Number	Permit From
Arid Recovery	58-2015	South Australian Wildlife Ethics Committee
St Peters Island	U26475-1	South Australian Wildlife Ethics Committee
Salutation Island	2016-34	Western Australian Department of Parks and Wildlife Animal Ethics Committee
Mt Gibson	2014-20	Western Australian Department of Parks and Wildlife Animal Ethics Committee

SI Table 2: Unique barcodes used for each double-digest RAD library. Shown here in 96-well plate format.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTCGAGT	GTGTGAGT	GAGTATG	GACTCT	AGACGAGA	CCGTCGCCA	GCAGA	CGAGAG	TCCGCCGCA	GCTCG	ACGATG	GTACGT
B	AGCACTA	TGCATGCGT	CATGGCCGA	CTATCG	GAACAAGT	ACACT	TGATCTA	AGGCGACCT	ACCTACCG	GTATTCTAT	CAGCAGA	GCCAT
C	GTCGATG	TCACGAGT	AATGGACA	ACTCGCCA	TCAGATG	TGGCAA	ATGTACT	CCGCGTCCA	ATCACTAT	ATCAGAG	CTCAT	TACTGATAG
D	GTCGTACTG	ATGGCG	CGTATGCT	CGAGCGT	TGCCACCA	TACTACAG	CCGGACTGA	GAGCGTCGT	TACGA	ATACACAG	CTGAAGAA	TTCCGA
E	TCTCTCA	CGTGATGCA	CACTGAG	TGCAGCG	AGTGACAA	ATCTGTCT	GTCTG	CAGTATAGA	TAGATGTA	GCATCAGCG	CATCCG	ATTCGG
F	TATGATTCT	TAGCGTG	AGCAGTCT	TCTTATAGT	GTACGCTGT	CTATA	TCACTG	GGCTAG	ATGTCA	GTGTGCAT	ACTACGTG	CGTCGA
G	CCGATGGCA	CACTCG	TGTATAT	TTGTTAACT	ACCTG	TATCT	AGTATAGT	TGTAG	TCATGG	TTAATCGTT	CGCGCATA	CTGCG
H	AGCTA	GTGCGATAG	ACGTGTACT	ACTCTA	GCTGACG	ACGAGTG	ATCTGTA	TGCTAT	TCGTACTA	CAGATA	TAGGCT	AGCCGTGCA

SI Table 3: Sequencing results of samples used in this study

Sample ID	Population	Reads	Number of Loci	Number of SNPs	Percent Missing Data	Average Depth of Coverage
321	Arid_Recovery	1747771	91034	6219	28.71	11.99
282	Arid_Recovery	2624274	116537	7229	17.13	16.74
284	Arid_Recovery	2722355	116790	7996	8.33	17.12
2197	Arid_Recovery	3258362	149041	7673	12.04	15.48
294	Arid_Recovery	5189201	170074	8500	2.56	24.50
347	Arid_Recovery	3576200	136011	7957	8.78	19.74
559	Arid_Recovery	3724920	156128	8314	4.69	17.88
430	Arid_Recovery	4361914	154409	8307	4.77	22.20
435	Arid_Recovery	2663562	112107	7906	9.37	16.39
646	Arid_Recovery	4750963	174597	8527	2.25	21.01
560	Arid_Recovery	3471059	144980	8191	6.10	17.73
493	Arid_Recovery	6622181	211613	8600	1.41	24.48
516	Arid_Recovery	8386909	234469	8640	0.95	29.24
597	Arid_Recovery	6077728	204073	8617	1.22	23.62
328	Arid_Recovery	3699057	148902	8262	5.28	18.50
513	Arid_Recovery	8031913	201222	8624	1.13	32.80
530	Arid_Recovery	4380176	168614	8362	4.14	19.86
LC1439	East_Franklin	2424943	110503	6763	22.47	14.86
LC1438	East_Franklin	1998033	93870	6327	27.47	15.07
LC1441	East_Franklin	6280569	221856	8591	1.51	21.72
LC1440	East_Franklin	3657452	149893	8091	7.25	17.49
LC1445	East_Franklin	3182399	146615	7952	8.84	15.42
LC1444	East_Franklin	4734105	185412	8384	3.89	18.87
LC1421	East_Franklin	5457258	195919	8577	1.67	20.98
LC1447	East_Franklin	4312959	170101	8327	4.54	19.05
6047	Monarto	1796858	90768	6332	27.41	13.57
6048	Monarto	2142067	102406	6974	20.05	14.54
6046	Monarto	2361385	109678	7179	17.70	15.49
6045	Monarto	2710666	121883	7856	9.94	16.00
6049	Monarto	2258007	93039	7485	14.19	17.36
6044	Monarto	4005091	144880	8207	5.92	21.21
77E8C54	Mt_Gibson	1902887	93857	6448	26.08	12.98
7A0149F	Mt_Gibson	13377179	274184	8643	0.92	41.15
7A3C855	Mt_Gibson	3037446	145336	7674	12.03	14.68
7A2E993	Mt_Gibson	5054695	195981	8341	4.38	19.73
74D8EA2	Mt_Gibson	4317118	169354	8507	2.48	18.86
74D970C	Mt_Gibson	9826289	253282	8659	0.73	31.57

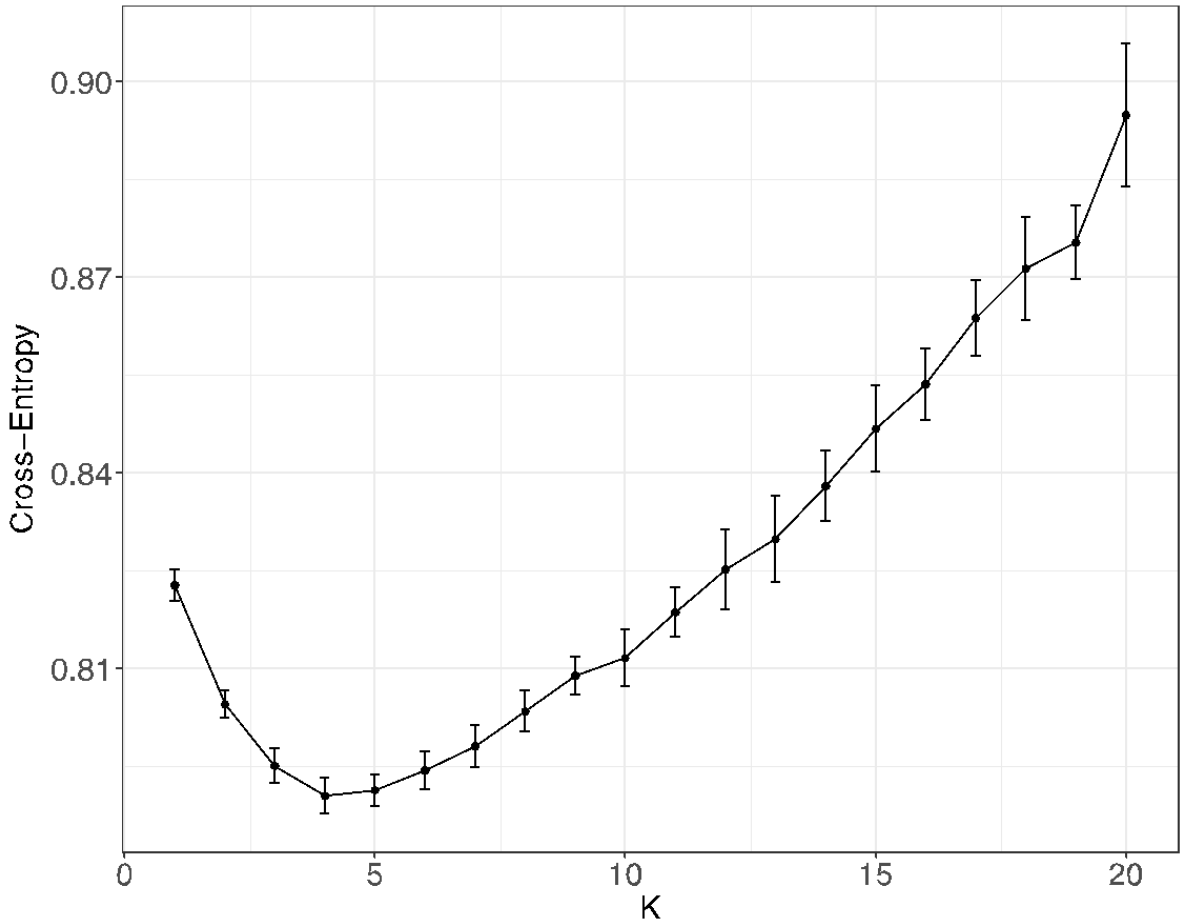
79F7AA0	Mt_Gibson	3193030	137322	8069	7.50	16.43
77E8974	Mt_Gibson	8200164	221372	8618	1.20	30.20
609A	Reevesby_Island	1586931	81627	5749	34.09	12.57
6011	Reevesby_Island	1810268	80475	6889	21.02	15.24
6306	Reevesby_Island	962517	45656	3977	54.41	13.14
6305	Reevesby_Island	1341994	71600	5156	40.89	11.86
6324	Reevesby_Island	928256	41942	3616	58.55	13.79
6019	Reevesby_Island	1109242	57961	5050	42.11	10.78
6313	Reevesby_Island	1552939	71955	5473	37.26	13.79
6017	Reevesby_Island	1374737	70029	5645	35.29	12.52
6331	Reevesby_Island	1323380	69248	6024	30.94	12.15
6006	Reevesby_Island	2121320	106741	7199	17.47	13.81
6332	Reevesby_Island	2229417	105942	7007	19.67	14.73
6327	Reevesby_Island	2752424	118017	7485	14.19	16.71
6328	Reevesby_Island	1668713	85246	6030	30.87	13.07
6031	Reevesby_Island	2657781	121889	7660	12.19	15.92
6003	Reevesby_Island	2720249	115613	7511	13.89	17.31
6314	Reevesby_Island	2629099	130001	7570	13.22	14.28
6319	Reevesby_Island	3504089	128088	7253	16.85	20.72
6042	Reevesby_Island	5846226	186009	8573	1.72	24.93
6020	Reevesby_Island	2146368	101834	7551	13.44	13.94
6009	Reevesby_Island	3933136	146984	8159	6.47	20.70
6308	Reevesby_Island	3418055	119844	7208	17.37	21.97
6041	Reevesby_Island	3502000	140178	8184	6.18	18.81
6337	Reevesby_Island	1492285	65974	4204	51.81	15.40
6325	Reevesby_Island	2430544	112683	7263	16.74	15.26
6010	Reevesby_Island	7235658	194627	8520	2.33	29.59
6338	Reevesby_Island	2264804	91728	6749	22.63	17.94
6033	Reevesby_Island	4395750	151217	8340	4.39	21.95
6304	Reevesby_Island	2439437	98427	7491	14.12	18.19
6035	Reevesby_Island	4338173	161604	8457	3.05	20.17
6039	Reevesby_Island	2776634	116126	7292	16.40	16.98
6016	Reevesby_Island	4429305	162149	8524	2.28	20.65
6309	Reevesby_Island	3664626	140247	7283	16.51	19.65
6326	Reevesby_Island	3506182	129863	7819	10.36	19.93
6301	Reevesby_Island	5957349	175793	8541	2.09	26.90
6018	Reevesby_Island	2512222	109692	7913	9.29	15.73
6334	Reevesby_Island	8585082	229603	8644	0.91	29.68
6322	Reevesby_Island	3147220	127573	7637	12.45	18.25
6333	Reevesby_Island	5485231	176274	8360	4.16	24.95
6037	Reevesby_Island	3306814	143647	8142	6.66	16.80
6001	Reevesby_Island	3562713	138711	7858	9.92	19.02
6329	Reevesby_Island	5241695	164683	8392	3.79	25.72

6318	Reevesby_Island	3749760	140996	8264	5.26	19.90
6302	Reevesby_Island	7946053	201326	8581	1.63	31.85
6023	Reevesby_Island	4735540	173311	8530	2.21	21.18
6310	Reevesby_Island	4343418	141167	7712	11.59	24.12
6330	Reevesby_Island	4670781	134939	8133	6.76	27.20
6317	Reevesby_Island	5069803	179199	8580	1.64	22.32
6021	Reevesby_Island	3342776	143707	8081	7.36	17.25
6002	Reevesby_Island	4822016	168566	8522	2.30	21.76
6339	Reevesby_Island	5186259	166083	8341	4.38	24.62
6014	Reevesby_Island	6516891	191091	8485	2.73	27.35
6022	Reevesby_Island	5063856	170874	8368	4.07	22.66
6038	Reevesby_Island	2980655	123523	8049	7.73	17.31
6312	Reevesby_Island	6712318	217950	8616	1.23	24.44
6040	Reevesby_Island	4844842	169300	8492	2.65	22.51
6036	Reevesby_Island	2727863	116844	8049	7.73	16.46
6027	Reevesby_Island	6672776	193934	8617	1.22	27.24
6004	Reevesby_Island	6874160	196716	8603	1.38	27.51
6015	Reevesby_Island	5279965	162513	8331	4.49	25.43
6335	Reevesby_Island	4463745	160044	8396	3.75	21.05
6043	Reevesby_Island	7014082	194629	8605	1.35	28.91
6341	Reevesby_Island	3221365	122277	8147	6.60	19.19
6311	Reevesby_Island	5743541	185296	8418	3.50	24.09
6007	Reevesby_Island	11527763	279400	8650	0.84	33.60
6026	Reevesby_Island	5361895	179446	8585	1.58	23.68
6303	Reevesby_Island	5164334	165274	8472	2.88	24.60
6024	Reevesby_Island	3830691	135381	8237	5.57	20.86
6030	Reevesby_Island	5978084	174431	8551	1.97	27.29
6340	Reevesby_Island	9380477	210428	8437	3.28	36.50
6323	Reevesby_Island	5776631	192029	8598	1.43	23.65
6320	Reevesby_Island	5991492	189046	8589	1.54	25.09
6315	Reevesby_Island	5797846	201079	8589	1.54	22.50
N15NH10	Salutation_Island	6028411	205372	8545	2.04	22.19
E2EB1	Salutation_Island	6666027	232229	8640	0.95	22.38
W2WB1	Salutation_Island	2175611	105402	7443	14.67	14.04
W1WG2	Salutation_Island	5131050	195268	8508	2.46	20.53
N28NI1	Salutation_Island	3025591	139815	7932	9.07	15.08
E5ED5	Salutation_Island	3329310	153833	7966	8.68	15.59
W3WD1	Salutation_Island	3954834	166399	8395	3.76	17.85
E4EA5	Salutation_Island	2881333	142489	7824	10.31	14.47
E3ED6	Salutation_Island	3841132	168735	8340	4.39	16.94
W8WE10	Salutation_Island	6498047	215426	8603	1.38	23.80
W7WC19	Salutation_Island	5176674	199259	8542	2.07	20.34
N6NC6	Salutation_Island	3807970	164631	8240	5.54	17.10

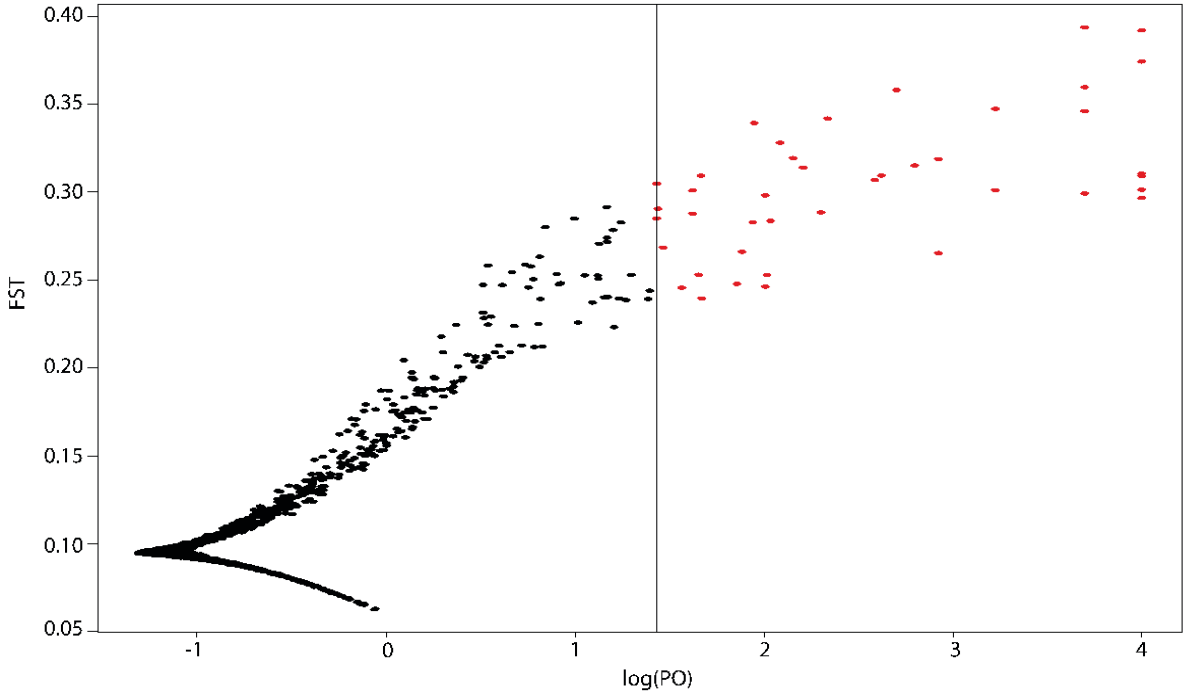
W5WF9	Salutation_Island	3479545	157681	8224	5.72	16.51
E7EF6	Salutation_Island	3689540	166486	8219	5.78	16.17
N49NI8	Salutation_Island	4704528	188456	8316	4.67	18.49
E1EC6	Salutation_Island	4478095	177991	8444	3.20	18.89
N45NE6	Salutation_Island	5450092	205758	8382	3.91	19.85
N52NJ4	Salutation_Island	2942568	135518	7827	10.27	14.91
N42ND8	Salutation_Island	7170245	249368	8631	1.05	22.86
SNR07	St_Peter	3706246	158380	8235	5.59	17.26
SNR04	St_Peter	2396512	109850	7669	12.08	15.49
SNR10	St_Peter	2753928	127082	7684	11.91	15.35
SNR11	St_Peter	2928129	139151	7742	11.25	15.28
SNR13	St_Peter	2894773	133032	7678	11.98	15.52
SNR12	St_Peter	2501466	118638	7714	11.57	14.32
SNR08	St_Peter	2695712	118689	7945	8.92	16.45
SNR02	St_Peter	13861969	298900	8648	0.86	39.10
SNR03	St_Peter	8946846	248072	8668	0.63	29.64
LC1414	West_Franklin	2542062	121235	7608	12.78	13.84
LC1419	West_Franklin	4581604	180037	8494	2.63	18.83
LC1428	West_Franklin	5833920	209259	8607	1.33	21.70
LC1416	West_Franklin	5682392	197233	8587	1.56	22.72
LC1437	West_Franklin	4068915	150302	8298	4.87	19.55
LC1450	West_Franklin	6616710	225660	8593	1.49	22.72
LC1417	West_Franklin	4603828	178858	8482	2.76	19.10

SI Table 4: Average within- and between- population pairwise relatedness

Population 1	Population 2	Avergae Pairwise Relatedness	Standard Deviation
Salutation Island	East Franklin	-0.2396	0.0455
Salutation Island	Arid Recovery	-0.2284	0.0361
Salutation Island	Monarto	-0.2079	0.0464
Salutation Island	Reevesby Island	-0.1968	0.0481
Salutation Island	Mt Gibson	-0.1966	0.0554
Salutation Island	West Franklin	-0.1836	0.0387
Salutation Island	St Peter Island	-0.1793	0.0504
Mt Gibson	St Peter Island	-0.1786	0.0647
Mt Gibson	East Franklin	-0.1732	0.0531
Arid Recovery	East Franklin	-0.1731	0.0398
Arid Recovery	Mt Gibson	-0.1703	0.0668
Reevesby Island	Mt Gibson	-0.1666	0.0624
Reevesby Island	East Franklin	-0.1650	0.0491
Arid Recovery	West Franklin	-0.1609	0.0362
Mt Gibson	Monarto	-0.1603	0.0523
West Franklin	East Franklin	-0.1501	0.0546
St Peter Island	East Franklin	-0.1498	0.0394
West Franklin	Monarto	-0.1470	0.0379
St Peter Island	West Franklin	-0.1450	0.0457
Reevesby Island	West Franklin	-0.1415	0.0367
Arid Recovery	St Peter Island	-0.1185	0.0399
Reevesby Island	Monarto	-0.1052	0.0622
Mt Gibson	West Franklin	-0.1046	0.0459
Reevesby Island	St Peter Island	-0.1023	0.0468
Arid Recovery	Monarto	-0.1006	0.0526
St Peter Island	Monarto	-0.0791	0.0623
Arid Recovery	Reevesby Island	-0.0729	0.0477
East Franklin	Monarto	-0.0627	0.1210
West Franklin	West Franklin	-0.0624	0.0271
East Franklin	East Franklin	-0.0622	0.0603
Reevesby Island	Reevesby Island	-0.0435	0.0551
Monarto	Monarto	-0.0391	0.1120
St Peter Island	St Peter Island	0.0034	0.0446
Arid Recovery	Arid Recovery	0.0907	0.1175
Salutation Island	Salutation Island	0.1530	0.0746
Mt Gibson	Mt Gibson	0.3311	0.1637



SI Figure 2: sNMF cross-entropy criterion plot. Dots and bars represent means and standard deviations calculated for each K value. The lowest mean (K=4) is taken as the most likely number of 'gene pools' (K).



SI Figure 3. Signatures of selection in the GSNR inferred using the program Bayescan. Each dot represents a locus. The vertical axis indicates mean FST between the 8 populations and the horizontal axis indicates the log posterior odds (PO). The vertical line indicates the false discovery rate threshold of 0.01. Loci to the right of this line (red dots) are putatively under selection

Chapter 7

Discussion and Conclusion

Chapter 7: Discussion and Conclusion

Thesis Summary and Significance

The aim of my thesis was to advance our understanding of species declines and extinctions, and to apply current knowledge in the area of extinction science to improve conservation outcomes for threatened taxa. I specifically focus on native Australian mammals, a unique and remarkable group that has suffered a disproportionate number of extinctions and catastrophic declines. I summarise my findings and discuss their significance and implications to conservation in Australia below.

Summary

In Chapters 2 and 3 I examined the timing of the devil (*Sarcophilus harrisii*) and thylacine (*Thylacinus cynocephalus*) mainland extinctions. In Chapter 2 I used genetic population assignment to show that devil individuals found recently in Victoria, Australia did not represent a relict population on the mainland, but instead were recent translocations from Tasmania. In Chapter 3 I validated the assumed synchronicity of the mainland devil and thylacine's extinctions by collating a large dataset of radiocarbon dates and applying inferential methods to estimate extinction time. These chapters provide a strong base on which further research into the devil and thylacine mainland extinctions can build and contribute to the discussion surrounding the suggested Australian re-wilding conservation projects, which propose to release native predators into mainland environments as a means of ecological restoration and feral-predator control.

In Chapter 4 I generated and analysed a dataset of ancient and historical thylacine mitochondrial DNA sequences, finding evidence of a population expansion in the Tasmanian

thylacine population that coincides with a decline in the Tasmanian devil population and a climate change event across Australia. This points to a possible ecological regime shift in Tasmania ~3,000 years before present and suggests that climate change has been undervalued as a driver of the thylacine and devils' mainland extinction. This result is important given the parallels between the changes occurring 3,000 years ago and changes occurring today. In both cases the combined effects of introduced species, climate change and human-driven habitat modification put stress on native species. As anthropogenic climate change is predicted to increasingly impact the Australian environment, our results emphasize the need to prepare and intervene in order to save threatened Australian native species from extinction.

In Chapters 5 and 6 I conducted high resolution genetic monitoring of four Australian mammal species, which are all part of ongoing reintroduction programs. In Chapter 5 I investigated genetic diversity and inbreeding across all extant populations of the greater stick nest rat (*Leporillus conditor)*, and in Chapter 6 I examined the change in genetic diversity since translocation in four species (the greater stick nest rat, the greater bilby [*Macrotis lagotis*], the burrowing bettong [*Bettongia lesueur*] and the western barred bandicoot [*Perameles bougainville*]) reintroduced to Arid Recovery reserve. My results from these chapters will be used to guide directly the conservation management of these populations, and provide a greater understanding of genetic change in reintroduced populations, which will be of interest to all future reintroduction programs of any species. Significantly, these chapters highlight the benefits of strategic sourcing of animals from slightly diverged populations to maximise the retention of genetic diversity – an area with scant published data on real-world threatened species.

Significance

Australian mammals have suffered a disproportionate number of extinctions and declines over the course of several extinction events. For example, the loss of the megafauna during the Pleistocene, the loss of the largest marsupial carnivores from the mainland during the late-Holocene, and finally and most significantly, the extraordinary number of extinctions and declines suffered since European arrival (Woinarski *et al.,* 2015).

Studying past mammal extinctions alerts us to true scale of extinction risks in altered environments and provides insight into the range of effects of species loss on the complex networks that make up ecosystems (Fordham *et al.*, 2016). Understanding genetic components of conservation projects is also an essential research area as it is well established that loss of genetic diversity is detrimental to a species long-term persistence, but how best to manage populations to mitigate genetic diversity loss and its negative effects is less clear (Frankham *et al*., 2010). A greater number of studies in these areas, such as those described in my thesis, will provide a greater ability to generalise findings and add to the collective pool of experience that conservation biologists and managers call upon to make policy recommendations.

Ultimately, the research presented in my thesis contributes to our understanding of the natural history of various Australian mammals with implications for broad and targeted conservation action in the Australian context. The remainder of this chapter focuses on some of the limitations I encountered during my research and discusses approaches that could be implemented in the future to overcome them.

Limitations and Future Directions

Some of the limitations that became apparent during my research are inherit to the types of datasets I generated, others are linked to the analytical approach used, while others still are issues more general to the fields of paleoecology, ancient DNA, conservation genetics. Below I discuss these problems and highlight the opportunities and future directions that could overcome them.

Fossil Discovery

Chapters 3 and 4 are based on the available fossil samples of mainland thylacines and devils. The results of both chapters would have been more precise if more samples from a greater variety of time periods and geographical locations were available. Because of limited availability of samples from eastern Australia, in Chapter 4 our ability to make demographic inferences about that population was limited and in Chapter 3 we only explored the extinction time of the species across its entire range (i.e. the whole of southern Australia). It is unlikely that extinction times of local populations of a species are exactly synchronous, and there is evidence that both the mainland devil (Brown, 2006) and thylacine (Chapter 4) had contracted into east/west groups prior to extinction. Increased sampling in eastern Australia is needed to resolve these issues.

However, the quality and number of samples in studies of extinct taxa depends not just on sampling effort (as with studies of extant taxa), but also on stochastic preservation biases – taphonomic bias (Benton *et al.*, 2011). Fossils for most taxa are rare, a limitation that affects Chapters 3 and 4, but that is also common to all paleontological studies.

A recent paper by Block *et al*. (2016) describes a new method to assist the discovery of fossils by modelling the estimated past distribution of species, the geological suitability for fossil preservation and the likelihood of fossil discovery in the field. The applications of such

methods could improve fossil discovery for all paleontological studies, but could also be used to extend methods of extinction time estimates such as those used in Chapter 3. For example, by modelling the likelihood of fossil preservation in geographical space, we could estimate the probability that the absence of fossils represents true absence of the species in a given area through time (F. Saltré *pers. comm.*). A greater understanding of the patterns of the mainland devil and thylacine decline in geographical space would further help to tease apart the mechanisms behind their extinctions.

Ancient DNA Preservation in Australia

It is well established that DNA preserves better in cold and dry environments and that average temperature through time is a good predictor of DNA survival. Hofreiter *et al.* (2015) built a model of DNA degradation based on environmental temperature, shown in Figure 1. This map gives an impression of the difficulties of working with ancient DNA from Australia.

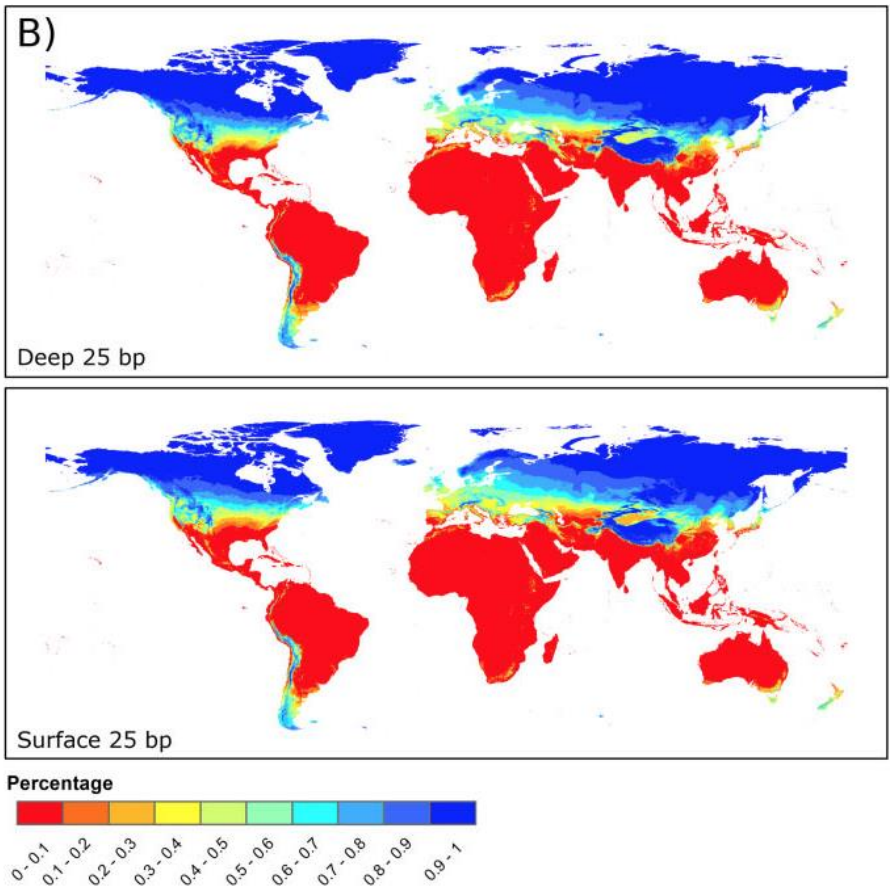


Figure 1. Modified from Hofreiter *et al*., (2015). Expected survival of DNA after 10,000 years of a 25 bp fragment.

In Chapter 4, to overcome the issues of poor DNA preservation in Australia, we used hybridisation enrichment and next generation sequencing to selectively capture and sequence short, low concentration endogenous DNA fragments (Llamas *et al.*, 2015). Additionally, we focused on the mitochondrial genome, which has a larger copy number per cell and therefore a greater chance of recovery from ancient specimens than nuclear DNA (Ho and Gilbert, 2010). Despite the use of these methods, we successfully recovered mitochondrial genome sequences with sufficient coverage and read depth from less than 50 % of ancient samples (> 600 years old) sequenced, compared to 90 % of historic samples (< 600 years old).

A recent aDNA study examining the quantity of endogenous DNA in various human bones found that the petrous bone in the inner ear had 4-16 times more endogenous DNA

than teeth and up to 183 times more endogenous DNA than other bones (Gamba *et al*., 2014). Petrous bones are the hardest and densest bones in the mammalian body and Gamba *et al*. (2014) suggests that the compact nature of petrous bones protects the DNA within them from post-mortem bacterial and chemical-mediated decay. Another study examined DNA preservation in petrous samples from several environments (Pinhasi *et al*., 2015), finding that while the percentage of endogenous petrous DNA from hot and humid regions was still low, it was detectable. Given these results, an examination of DNA preservation in petrous bones in the Australian context, and in species other than humans, would be useful to future ancient DNA studies in this region.

Limitations of Single Loci

In Chapter 4 we used mitochondrial DNA to reconstruct the thylacine's phylogeographic and population history in Australia. Mitochondrial DNA (mtDNA) is well suited to aDNA studies (see above section) and has several characteristics that make it amendable to genetic analysis, including maternal inheritance, absence of recombination and high mutation rate (Ramakrishnan and Hadly, 2009). However, the use of a single locus, such as mtDNA, to reconstruct demographic history is challenging and, as I found in chapter 4, can produce large confidence intervals that limit the usefulness of the inference (Heled and Drummond, 2008). This is because a single locus only represents one realization of the coalescent process, resulting in considerable error in the estimates of effective population size.

Demographic estimates can be substantially improved by using multiple independent loci (Gill *et al.*, 2013; Heled and Drummond, 2008). Increasing the number of unlinked loci in coalescent analyses allows the uncertainty in the coalescent to be assessed, leading to an

improvement in the reliability of the demographic inference and a substantial reduction in estimation error (Ho and Shapiro, 2011).

Improved inference can also be gained by generating whole genome sequences, which allows the implementation of methods such as PSMC (pairwise sequentially Markovian coalescent; Li and Durbin, 2011) and MSMC (multiple sequentially Markovian coalescent Schiffels and Durbin, 2014) to infer demographic history. These methods use the entire collection of loci across the genome (or multiple genomes in MSMC) to calculate the most recent common ancestors for windows of allele pairs. Since the rate of coalescence is inversely proportional to effective population size, effective population size through time can be inferred. For example, when many loci coalesce at the same time, it is a sign of small population size at that time.

Recovering nuclear loci or whole genome sequence data from ancient specimens is more challenging than recovering mtDNA, requiring greater sequencing depth because of the lower copy number and greater sequence length. Shot-gun sequencing can therefore be cost-prohibitive for these types of samples. Hybridisation enrichment can be used to reduce the depth of sequencing necessary to recover nuclear data from highly degraded samples. This could be achieved for thylacines using exon baits designed for marsupials broadly (Bragg *et al*., 2016). Targeting the thylacine genome more specifically would require a reference genome of the target species (or a close relative) from which to design baits (Carpenter *et al*., 2013; Enk *et al.*, 2014; Horn, 2012). The Tasmanian devil diverged from the thylacine approximately 40 million years ago (Mitchell *et al*., 2014) and has had its genome assembled (Epstein *et al*., 2016). Previous work has shown that cross-species hybridisation enrichment can be successful with low to moderate amounts of sequence divergence (Jin *et al*., 2012; Portik *et al*., 2016) between baits and target and thus the Tasmanian devil genome may provide the necessary genomic resources to design baits for thylacine samples. Future work

on the demographic history of the thylacines would benefit from the investment that the generation of a reference genome.

Paleo-Disease

It has often been suggested that disease may have contributed to the thylacine's Tasmanian extinction, as well as the devil and thylacine's mainland extinction (Guiler, 1985; Paddle, 2002). Anecdotal evidence of a 'distemper-like' disease in Tasmanian thylacines (Paddle, 2012) and the recent outbreak of the devastating devil facial tumour disease (DFTD) in the extant Tasmanian devil population (Epstein *et al*., 2016) give some credence to the 'disease hypothesis.' Prowse *et al*. (2013) used population viability analysis (PVA) to show that a disease scenario is not necessary to explain the Tasmanian thylacine's extinction, although its inclusion in the models increased their extinction risk slightly.

In Chapters 3 and 4 we are unable to test the disease hypothesis for either the devil and thylacine mainland extinctions or the thylacine's Tasmanian extinction because traces of disease pathology are not (usually) recorded in the fossil record or in an organisms preserved DNA (Kathleen Lyons *et al.*, 2004).

A possible avenue to explore the disease hypothesis in devils and thylacines (and other extinct taxa) is by using the developing methods of metagenomics. Metagenomics refers to the technique of characterizing genetic data of whole communities of organisms rather than an individual species (Tringe and Rubin, 2005). Due to the revolution of next generation sequencing, it is now possible to sequence nearly every molecule in a DNA extract. Metagenomic studies exploit this feature of NGS to characterise genetic data from every organism in a sampled community (Eisen, 2007). Metagenomics has been used to identify and sequence the genomes of microbial organisms, including pathogens, in numerous ancient human specimens from bone, tooth and mummified tissue samples (Donoghue, 2013). The

field is still in its infancy and has rarely been applied to non-human ancient specimens. However mummified thylacines from the Nullarbor region in Western Australia (Lowry and Merrilees, 1969), or well preserved museum specimens from Tasmania, may be viable candidates.

Limitation of RAD-seq

Restriction site-associated DNA sequencing (RAD-seq) has become a popular method of data generation for molecular ecology studies. In Chapters 5 and 6, I used double digest RAD-seq (ddRAD-seq; Peterson *et al.*, 2012; Poland *et al.*, 2012), which eliminates the costly random shearing step of other methods and can be customized to recover hundreds, to hundreds of thousands of loci.

A major drawback of ddRAD-seq is the use of a PCR amplification step, which may introduce PCR artefacts in the final sequencing dataset (Schweyen *et al.*, 2014). PCR duplicates, the most common form of artefacts, can skew allele frequency estimates, potentially leading to false genotype calls (Pompanon *et al.*, 2005). Unfortunately, PCR duplicates are impossible to identify bioinformatically in ddRAD-seq datasets due to the non-random digestion step, which makes all homologous DNA fragments from multiple genome copies in an extract the same length, meaning they are indistinguishable from PCR duplicates. Recently however, several studies have overcome this limitation by including a stretch of random, degenerate bases in the adapter sequences ligated to the digested DNA fragments, which allows *in silico* identification of PCR duplicates (Franchini *et al.*, 2017; Schweyen *et al.*, 2014; Tin *et al.*, 2015). The adoption of this method will increase confidence in genotype calls from ddRAD-seq projects.

A further challenge of using RAD-seq methods is accounting for biases introduced during bioinformatic processing. Several pipelines have been developed to process raw RAD-

seq data, by either mapping reads to a reference genome or by *de novo* assembling them into putative orthogolous loci (Catchen *et al*., 2013; Eaton, 2014; Puritz *et al*., 2014). In Chapters 4 and 5 I used the industry standard pipeline STACKS (Catchen *et al*., 2013) to *de novo* assemble loci, as reference genomes were not available for my study species. Very few studies have examined how different pipelines and parameters can influence downstream biological inferences. One to do so recently found that a number of summary statistic were affected by pipeline choice, but that STACKS was generally the most appropriate for *de novo* assembly (Shafer *et al*., 2016). The study also found that mapping reads to a reference genome (as opposed to *de novo* assembly) greatly improved accuracy and robustness of the statistics (Shafer *et al*., 2016). This indicates that where possible, RAD-seq studies should use reference-based approaches and that conservation genetic studies will be improved as the number of available reference genomes increases.

Critical levels of inbreeding

Currently one of the greatest unknowns in conservation biology is how we translate measured estimates of genetic inbreeding to estimates of biologically meaningful inbreeding depression in wild populations of threatened species. In Chapters 5 and 6 I measured inbreeding and genetic diversity in reintroduced populations of several species to guide conservation management. I tested whether inbreeding was significantly higher in reintroduced populations compared to source/founder groups to indicate whether assisted gene flow should be considered in reintroduced populations. This threshold is subjective as, without additional information, it is not possible to say what level of inbreeding will cause inbreeding depression.

Evidence of inbreeding in wild population typically comes from observed decreased survival (most often measured at the juvenile life-stage) and can be expressed using the

measure of lethal equivalents (Keller and Waller, 2002). A lethal equivalent is defined as a group of detrimental alleles that would cause, on average, one death if homozygous (e.g. one lethal allele or two alleles with 50% probability of causing death when homozygous). The number of lethal equivalents is estimated from the slope of the regression of natural log of survival on the inbreeding coefficient (F; Frankham *et al.*, 2010). While detrimental effects of inbreeding on adult traits (such as fecundity, longevity, offspring birth weight and milk production) are well known in agricultural and captive populations (Leroy, 2014; Ralls *et al.*, 1988), their prevalence and magnitude in wild populations is unclear (although see Grueber *et al.*, 2010; Szulkin *et al.*, 2007 for examples from two bird species).

Average measures of lethal equivalents have been published for captive mammals (Ralls *et al.*, 1988) and wild mammals and birds (O'Grady *et al.*, 2006). However, inbreeding depression in captive environments is known to be a poor proxy for inbreeding depression in the wild (Armbruster and Reed, 2005) and lethal equivalents estimated for wild populations are limited in number and are extremely varied (Frankham *et al.*, 2014). Thus, to accurately estimate lethal equivalents for a population (and thereby the amount of inbreeding depression expected for a given level of inbreeding), data on survival, preferably at multiple life-stages, as well as accurate measures of inbreeding coefficients are needed.

It would be useful to the conservation management of my study species in Chapters 5 and 6 to conduct field experiments and monitoring to estimate the number of lethal equivalents in each species and population. A greater understanding of how varying levels of inbreeding translate to negative effects for population growth and survival would lead to more informed conservation decisions and greater efficiency in the management programs of the threatened species examined here. The Arid Recovery reserve is an excellent candidate for this approach as there are permanent staff on site and on-going monitoring and research projects for all four species examining life-history.

Characterising Adaptive Diversity and Selection

In genetic monitoring studies measured neutral genetic diversity is often used as a proxy for adaptive diversity in populations (Frankham *et al*., 2010). However, several studies have shown low correlation between neutral markers (such as microsatellites) and quantitative traits (Reed and Frankham, 2001). SNP markers like those used in Chapters 4 and 5 are expected to be better proxies than microsatellites for adaptive variation as a portion of markers are likely to fall within genes or other functional genetic regions (Helyar *et al*., 2011). However, delineating adaptive markers from neutral ones in SNP datasets is difficult (Bierne *et al.*, 2013).

Fst-outlier methods, such as those used in Chapters 4 and 5, are often used to detect possible functional SNPs under selection (Foll and Gaggiotti, 2008). We identified some SNPs in Chapter 5 putatively under diversifying selection among the stick-nest rat populations. Unfortunately, I was unable to identify what gene, or genomic region the putatively selected SNPs are linked to. This could be overcome by generating reference genomes to map SNP loci to (Oleksyk *et al*., 2010). Furthermore, by using whole genome sequencing or very dense SNP datasets (> a hundred thousand SNPs) mapped to a reference genome, footprints of selective sweeps, or the hitchhiking of neutral variants that are linked to the selected variant, could be detected, improving the identification of selected genomic regions (Bigham *et al.*, 2010).

However, as discussed in Chapters 5 and 6, demographic effects can confound Fst-outlier methods (Bierne *et al*., 2013; Hermisson, 2009). Adaptive selection can only be definitively identified using experimental approaches (Ballentine and Greenberg, 2010; Stapley *et al.*, 2010). For example, local adaptation could be tested for by measuring and comparing survival of animals sourced from sites that are ecologically similar and dissimilar

to the site to which they were reintroduced. If local adaptation has occurred, we would expect that the group sourced from the site more ecologically similar to the reintroduction site would have greater survival and fitness. Understanding the effects of local adaptation would assist the conservation management of many species, including those studied in Chapters 5 and 6 . For example, by validating genetic identification of selection and recognizing populations that are inappropriate as sources for reintroductions to certain areas.

Conclusion

My thesis contributes to our understanding of past extinction dynamics and current extinction risk in Australian mammals and provides analyses that will improve conservation outcomes for the species studied here and beyond. Through my research I encountered challenges, limitations and additional questions that I was unable to answer during my candidature. However, new questions that have been raised and limitations encountered simply provide exciting opportunities for future studies.

For example, re-estimating mainland thylacine and devil extinction times as new fossil are discovered will increase the power of this analysis, as will refining the statistical methods to include a spatial aspect. Similarly, including more ancient DNA sequences would help clarify the thylacine's phylogeographic structure on the mainland and focusing on petrous bones in the future may increase the likelihood of retrieving nuclear DNA and thus more robust estimates of demographic history. Additionally, taking advantage of innovations in the field of metagenomics to address the untested disease hypothesis for the thylacine and devil extinctions may help clarify a long-standing question surrounding these infamous species.

A multi-disciplinary approach that includes field experiments and monitoring, will be needed to address questions about the likelihood of inbreeding depression and whether local adaptation is occurring in the reintroduced populations studied here. Such experiments involve a large input of time and money, but have broad and significant relevance to conservation globally.

Finally, generating a reference genome for non-model species has multiple benefits for a range of studies. A reference genome would allow baits to be designed to multiple nuclear loci or whole genome sequences for use with ancient and museum specimens. This would allow a more refined reconstruction of the thylacine's demographic history. Additionally, using reference-based approaches in the assembly of RAD-seq loci will increase confidence in genotype calls, and will allow the identification of genes and genomic regions putatively under selection. The utility of reference genomes is well recognized, as evidenced by the recent announcement of the Earth BioGenome Project, which has the ambitious goal of sequencing the genomes of more than a million eukaryotic organisms (Pennisi, 2017). Such efforts to increase the number of reference genomes available will improve many studies across the sciences, including within the fields of extinction science and conservation. These issues are currently being addressed through the Oz Mammal Genomes Project (http://www.bioplatforms.com/oz-mammals/)

The Earth's biodiversity is being lost at an alarming rate, and we must act urgently to preserve it. Science has a large role to play in conservation as the better we understand species, ecosystems and the risks that threatened them, the better we will be able to protect them. I hope that the findings presented here, and the pursuits that arise from them in the future, continue to contribute to species conservation in Australia and beyond.

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Appendix I

Improving genetic monitoring of the northern hairy-nosed wombat (*Lasiorhinus krefftii*)

This appendix contains the publication describing the microsatellite markers I developed during my honours year to improve the genetic monitoring of the critically endangered northern hairy-nosed wombat. This article has been published in the *Australian Journal of Zoology.*

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Improving genetic monitoring of the northern hairy-nosed wombat (*Lasiorhinus krefftii*)

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Abstract. The endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*) has been monitored via remote sampling and genetic techniques since 2000, thus avoiding the detrimental effects on the animals of trapping and increasing the precision of abundance estimates. The currently available dinucleotide microsatellite markers used for this task are prone to stutter and other polymerase chain reaction artefacts, making allele calling difficult, and requiring costly duplication to ensure accuracy. To remedy this we have developed eight new tri- and tetranucleotide microsatellite markers that reduce the problem of stutter in DNA analysis. These new markers, along with three of the existing markers (two microsatellites and the SRY gender marker) were optimised in a single multiplex reaction that will reduce the time and cost of future northern hairy-nosed wombat hair censuses. We tested this new multiplex on 277 non-invasively collected hairs. One locus was rejected due to null-allele issues. The remaining nine microsatellite loci had two or three alleles. Genotype frequencies in the sample of detected individuals did not differ significantly from Hardy–Weinberg equilibrium and there was no evidence of linkage disequilibrium. This new multiplex provides comparable power to distinguish individuals, fewer issues with stutter artefacts and a reduced time and cost of analysis. It will be useful for future population censuses and long-term monitoring of individuals once they have been scored in previously genotyped and assigned samples.

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Introduction

The northern hairy-nosed wombat (*Lasiorhinus krefftii*) is a large, endangered, herbivorous, burrowing marsupial whose small population size and limited geographic range put it at risk of extinction (Horsup 2004). The species' range contracted dramatically during the 20th century as a result of competition with cattle, drought, and severe habitat loss leading to population fragmentation (Crossman *et al*. 1994). By 1981 the population was restricted to a few square kilometres at Epping Forest National Park in central Queensland and as few as 25 individuals remained (Gordon *et al*. 1985). Subsequently, the habitat has been highly managed and the population regularly monitored.

Conventional methods of population monitoring, such as live trapping, can be imprecise, costly, labour intensive and detrimental to the animals (Buckland 1993; Crossman *et al*. 1994; Arnemo *et al*. 2006). To overcome these problems, non-invasive genetic sampling has been used to estimate population size and sex ratio every 1–3 years through DNA analysis of remotely collected hairs (Banks *et al*. 2003). These genetic censuses have confirmed the positive effect of management actions and a steady increase in population size over the last 15 years.

These studies have used a subset of the most variable of 28 polymorphic microsatellite markers developed for the species by conventional means (Beheregaray *et al*. 2000; Walker *et al*. 2009). All but one of these is composed of dinucleotide repeats, targeted due to their abundance and technical convenience of isolation, one aspect of which was that selective screening of high-repeat-number (and therefore likely more variable) loci was formerly more straightforward for dinucleotides than for other repeat-motif types (Rassmann *et al*. 1991). This was an important consideration for a bottlenecked species like the northern hairy-nosed wombat (Taylor *et al*. 1994). Unfortunately, high-copy-number dinucleotide repeat markers are prone to slippage during polymerase chain reaction (PCR) amplification (Murray *et al*. 1993). This produces stutter peaks on acrylamide gels and chromatograms that in some cases make analysis and interpretation difficult, and necessitate duplication to ensure genotyping accuracy. In addition, the overlapping size ranges among the existing markers prevents them from being analysed

in a single multiplex reaction, adding additional time and cost to genetic analyses. Although this might be rectified to some extent through primer redesign, these markers will always be prone to stutter.

Scoring difficulties were particularly apparent during the 2010 census when genotyping was conducted for the first time using fluorescently labelled primers and capillary electrophoresis detection (J. Austin, unpubl. data), and in a new laboratory: only five of the seven core loci could be amplified reliably and stutter peaks were a significant problem in three of these. The lack of robustness of the existing marker system to changes in the analytical environment, and the requirement for PCR duplication to ensure accuracy in the face of slippage artefacts are undesirable features in the ongoing use of these markers for censusing the northern hairy-nosed wombat population.

Here we describe the isolation and characterisation of eight new polymorphic tri- and tetranucleotide microsatellite markers for the northern hairy-nosed wombat using next-generation sequencing (NGS). Tri- and tetranucleotide markers are less prone to slippage events, and therefore stutter, than dinucleotide microsatellites (Murray *et al.* 1993; Chakraborty *et al.* 1997; Schug *et al.* 1998). Allele drop-out and false alleles will still need consideration; however, reducing stutter will reduce scoring errors and improve the overall accuracy of genotyping. This approach allows for easy selection of a large number of such loci (which typically do not suffer stutter problems), and for which primer design can be optimised for multiplexing. These new markers, along with two of the original loci (Lla68Ca and L12: Beheregaray *et al.* 2000) and the SRY-gender marker, were incorporated into a single multiplex reaction. The efficacy of this new multiplex was tested by genotyping 277 non-invasively collected hair samples.

Methods

To rapidly isolate new microsatellite loci we used NGS following Gardner *et al.* (2011). Genomic DNA was extracted from muscle tissue of a single northern hairy-nosed wombat and sequenced on 1/16 of a PicoTiter plate using the Roche GS FLX (454) system (Margulies *et al.* 2005). Using the default settings of the program QDD2, all sequences were screened for a range of di-, tri-, tetra- and pentanucleotide repeats (Meglecz *et al.* 2010). The program Primer3, which is integrated in the QDD2 software, was used to design primer sets for all identified microsatellite loci (Rozen and Skaletsky 1999). From this list, loci were selected that met the criteria of Gardner *et al.* (2011): no homopolymers in the flanking region or primer binding site, no other microsatellites in the flanking region, no nanosatellites in the primer or flanking region, no compound microsatellites and no dinucleotide repeat motifs (due to their known association with PCR error and stutter: Guichoux *et al.* 2011).

Selected loci were tested for amplification success and polymorphism on DNA extracted from four northern hairy-nosed wombat tissue samples and 18 hair samples. PCR reactions were performed in a total volume of 10 μL containing 0.2 U Hotmaster Taq (Eppendorf, Hamburg, Germany), 1× Hotmaster buffer (Eppendorf, Hamburg, Germany), 0.2 mM each dNTP, 0.4 μM foward primer, 0.4 μM fluorescently labelled (6-FAM) reverse primer and 1 μL sample DNA. PCR amplification profiles consisted of 94°C for 2 min followed by 30 cycles of 94°C for 20 s, 55°C for 10 s, 65°C for 30 s, and then 65°C for 10 min. A small aliquot of each reaction was checked for the existence of the correct-sized product on 3.5% agarose gels stained with GelRed (Biotium, Hayward, USA) before the remainder was purified using the Qiagen MinElute 96 UF Purification Kit (Qiagen, Hilden, Germany) and diluted in water, to 1 : 50 for the tissue samples and 1 : 10 for the hair samples. The dilutions were sent to the Australian Genome Research Facility (Adelaide, Australia) where fragments were separated via capillary electrophoresis on an Applied Biosystems 3730 DNA analyser and analysed using AB Genemapper software.

A multiplex reaction incorporating polymorphic markers that amplified consistently across the 22 test samples was designed in the program Multiplex Manager (Holleley and Geerts 2009) using default parameters, except that the minimum distance between loci on the same dye was set to 15 base pairs (bp) (Fig. 1). Two previously developed markers that suffer only minimal stutter artefacts (Ll2 and Lla68CA: Beheregaray *et al.* 2000) and the SRY-gender marker (Liu *et al.* 2012) were also included in the multiplex design.

The effectiveness of this multiplex was assessed by genotyping 277 non-invasively collected hair samples from Epping Forest, 94 of which were randomly selected to be analysed twice to produce an estimated error rate. PCRs were performed using the Qiagen Type-it Multiplex PCR Kit (Qiagen, Hilden, Germany) in 12.5-μL volumes with 1× Type-it Master Mix (Qiagen, Hilden, Germany), 0.2 μM of each primer (Table 1) and 2 μL sample DNA (extracted and stored in Chelex following Sloane *et al.* 2000). Thermocycling consisted of 95°C for 5 min followed by 28 cycles of 95°C for 30 s, 60°C for 3 min, 72°C for 30 s, and then 60°C for 30 min. Products were purified using the Qiagen MinElute 96 UF Purification Kit (Qiagen, Hilden, Germany) and diluted 1 : 10 in water before capillary electrophoresis, as described above.

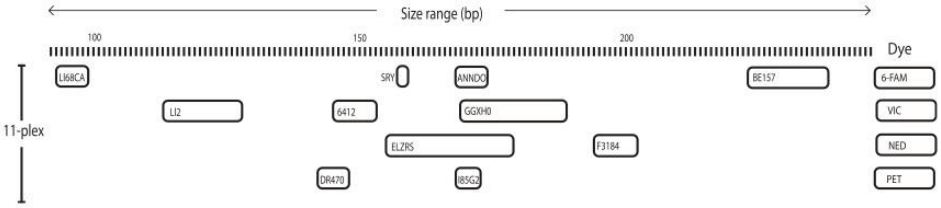

Fig. 1. Modified output from multiplex manager. Shows the dye assignment and allele size ranges of the designed northern hairy-osed wombat multiplex including the eight new and two previously designed microsatellite markers and the SRY-gender marker.

Table 1. Characteristics of 10 polymorphic microsatellite loci for the northern hairy-nosed wombat (*L. krefftii*)
No. of alleles (N_A), along with observed (H_O) and expected (H_E) heterozygosities and *P*-values of Hardy–Weinberg equilibrium tests (H–W(P)) are shown from *N* number of samples

	Primer sequences	Repeat motif	Size range (bp)	Dye label	*N*	N_A	H_E	H_O	H–W(P)	GenBank Acc. No.
ANNDO	GGACACAGCAGGAGAAAGCA CCTCCCATATTGTAAATATAGTCTCAT	$(AAC)_{11}$	168–174	6-FAM	99	2	0.135	0.146	0.44	KJ146614
BE157	CCATTGGAGGCAGAACAAAG CCTCTGAGGTAGGCAGGGTA	$(ACT)_{11}$	223–238	6-FAM	99	3	0.445	0.433	0.18	KJ146615
6412	GTTCCTTGAGAGGAGGGACC TTGCCTGGACAAGTGAATGA	$(AAGT)_9$	145–153	VIC	99	2	0.442	0.595	0.24	KJ146613
GGXHO	GGAGGACTTCAACCACAACC TGAAGAGCTTTCCTTTGAACCA	$(AGAT)_{22}$	169–185	VIC	99	3	0.514	0.485	0.58	KJ146620
ELZRS	TGCCACTTCATTTGGTCAAT ATTGGATGCACCTGGAAATG	$(AAT)_{15}$	155–179	NED	99	2	0.500	0.521	0.68	KJ146618
F3184	CCAAGAGCCAGAATGGTGAT AGCAGGCAAAGGATTGTGTC	$(AAGT)_{13}$	194–202	NED	99	2	0.050	0.052	0.79	KJ146619
DR470	AAACTCTGTCTTCTTGTTCAGGC GACTGCTCAGGTCCTAAATGGA	$(AAC)_{14}$	142–148	PET	99	2	0.500	0.411	0.09	KJ146617
I85G2	GTAGAAGGTGAGCGCCTTGA GGAGAGCAGACCTTGGGATT	$(AAGT)_{10}$	168–172	PET	99	2	0.486	0.479	0.89	KJ146621
LL68CA[A]	TCCTTTCCCACTCCACCAGC GAAGCATTACCATCGGAAAACC	$(CA)_{20}$	93–99	6-FAM	99	3	0.650	0.638	0.62	AF185106
LI2[A]	TGGTGCTCCTTTGATATCCC CCTGGGTCAGTGGCTTTG	$(CA)_{19}$	115–127	VIC	99	2	0.467	0.489	0.65	AF191296
Mean						2.3	0.419	0.415		

[A]Previously developed loci (Beheregaray *et al.* 2000).

Table 2. P(ID) and P(ID)SIB scores calculated after the addition of one marker at a time in order of decreasing variation

Locus added	No. of loci included	P(ID)	P(ID)SIB
LI68CA	1	0.2019	0.4780
GGXHO	2	0.0646	0.2682
ELZRS	3	0.0243	0.1595
DR470	4	0.0091	0.0949
I85G2	5	0.0035	0.0573
L12	6	0.0014	0.0350
BE157	7	0.0005	0.0217
6412	8	0.0002	0.0137
ANDDO	9	0.0002	0.0121
F3184	10	0.0001	0.0116

Table 3. Characteristics of nine new microsatellite loci in the southern hairy-nosed wombat (*L. latifrons*)
No. of alleles (N_A), along with observed (H_O) and expected (H_E) heterozygosities are shown from *N* samples

Locus	Product size range (bp)	*N*	N_A	H_E	H_O
GGXH0	131–191	16	7	0.64	0.38
DR470	125–155	16	4	0.65	0.69
DHZ50	135–151	16	4	0.66	0.63
I85G2	161–177	16	4	0.61	0.5
6412	153–161	16	3	0.58	0.53
ELZRS	157–166	16	3	0.33	0.38
F3184	173–185	16	3	0.58	0.63
BE157	222–225	16	2	0.23	0.25
ANNDO	159	16	1	0	0
Mean			3.44	0.47	0.44

Unique genotypes were identified in GIMLET (Valiere 2002). Chromatograms of all samples that mismatched by only one allele were visually reinspected to ensure that scoring errors had not led to false declarations of new individuals. In addition, 24 pairs of samples that mismatched by only one allele were included in the repeat genotyping error-rate estimation. The subset of unique genotypes was then checked for null alleles using the program MICRO-CHECKER (Van Oosterhout *et al.* 2004). After the removal of loci for which null alleles were suggested, unique genotypes were reidentified and ARLEQUIN 3.5 (Watson *et al.* 1998) was used on the new sample of detected individuals to estimate expected (H_E) and observed (H_O) heterozygosity, number of alleles (N_A), linkage disequilibrium (LD) and Hardy–Weinberg proportions. All results for multiple tests were adjusted using Bonferroni's correction (Rice 1989).

To quantify the power of the marker set to distinguish individuals, probability of identity scores ($P_{(ID)}$ and $P_{(ID)SIB}$), which estimate the probability that two random individuals or two full siblings will have identical genotypes, were calculated using the program GIMLET (Woods *et al.* 1999; Nicholls *et al.* 2000; Waits *et al.* 2001). Finally, sex linkage was checked by

examining the sex of heterozygote genotypes at each locus: if a marker is sex linked, males should express only one allele.

To assess the cross-species utility of the new markers we also genotyped 16 southern hairy-nosed wombat (*Lasiorhinus latifrons*) samples using single-plex reactions as described above. Southern hairy-nosed wombats have also suffered dramatic population fragmentation and declines and are the subject of conservation management programs, including population genetic monitoring (Walker *et al*. 2006).

Results

The single 1/16 plate Roche 454 NGS run produced 70403 reads, from which QDD2 mined 1820 putative microsatellites with flanking regions available for primer design. From these, 30 loci met the criteria of Gardner *et al*. (2011) and were therefore selected for PCR trial. Nine loci were found to be polymorphic, including four trinucleotide and five tetranucleotide repeat loci, all of which were included in the new multiplex design, along with two previously designed markers and the SRY-gender marker.

This multiplex was then used to genotype 277 samples. Subsequent quality checking in MICROCHECKER identified one locus (DHZ50) as potentially containing null alleles and it was thus excluded from downstream analysis. From the 277 samples analysed with the remaining markers, 97 unique genotypes were identified, of which 49 were male and 48 female. A per-locus error rate of 0.2% was estimated from mismatches between duplicate genotypes. This error rate is similar to that reported in previous non-invasive genetic studies of the northern hairy-nosed wombat (Sloane *et al*. 2000; Banks *et al*. 2003). Seventy-four pairs of samples differed by only one allele. Twenty-four pairs were regenotyped as part of the 94 randomly chosen for error checking, with no errors found. The number of alleles per locus was two or three. Observed (H_O) and expected (H_E) heterozygosity ranged from 0.052 to 0.638 and 0.050 to 0.650, respectively (Table 1). All loci were in Hardy–Weinberg equilibrium (adjusted $P = 0.0045$) and no significant LD was detected following Bonferroni correction (adjusted $P = 0.0006$). For every locus, multiple heterozygote male genotypes were observed, indicating that all are autosomal.

The suite of 10 microsatellite markers had probability of identity scores of 0.0001 and 0.0116 for unrelated ($P_{(ID)}$) and full-sibling pairs ($P_{(ID)SIB}$), respectively. P(ID) and P(ID)SIB scores calculated after removing one marker at a time in order of increasing variation are shown in Table 2.

Eight of the nine new markers were also found to be polymorphic in the southern hairy-nosed wombat after single-plex analysis of 16 samples, none of which had matching nine-locus genotypes. The nine new markers revealed an average of 3.44 alleles amongst these 16 individuals and expected and observed heterozygosity of 0.47 and 0.44 respectively (Table 3).

Discussion

Our results reiterate how NGS and associated bioinformatics are fast and effective tools for microsatellite discovery (Abdelkrim *et al*. 2009; Yu *et al*. 2011; Carvalho *et al*. 2012). The large amount of sequence data obtained from sequencing a portion of the northern hairy-nosed wombat genome allowed fast and cost-effective isolation of a wide variety of microsatellite loci, without *a priori* selection of repeat type or sequence. A subset of the most suitable markers could then be selected, using stringent quality filters, for subsequent testing. Nine new polymorphic microsatellite loci were isolated for the endangered northern hairy-nosed wombat that, in addition to two previously designed markers and the SRY-gender marker, were optimised for use in a single multiplex reaction.

Compared with the previous marker set applied to the same samples (J. Austin, unpubl. data), genotyping using the new marker set was faster, simpler and cheaper (due to the single-tube multiplex format), and was virtually free of stutter problems and artefacts. The newly designed markers all contain tri- and tetranucleotide repeats, rather than dinucleotide repeats as in the previously used northern hairy-nosed wombat microsatellite loci. This considerably reduced the amount of stutter produced, which simplified allele calling. Furthermore, the power of the new marker set to distinguish individuals is comparable to previous estimates using the original dinucleotide markers (Sloane *et al*. 2000).

By recalculating the P(ID) scores after removal of one marker at a time in order of increasing variability, a minimum number of markers for any chosen P(ID) score can be found. The typing of the eight most variable loci from the new marker set would produce similar scores as the typing of all 10 (Table 2).

The future of the northern hairy-nosed wombat is highly precarious and the species is very susceptible to several threats that could cause extinction. Continued monitoring of the species is vital to assist conservation efforts. The new markers and multiplex developed here will allow future censuses to be conducted more easily, at a reduced cost and in a much more timely fashion, than previously. They also represent an important resource for other types of studies of both northern hairy-nosed and southern hairy-nosed wombats that involve relatedness, paternity analysis, genetic diversity and population structure analysis.

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Appendix II

Distinctiveness of Pacific Robin subspecies in Vanuatu revealed from disparate patterns of sexual dichromatism, plumage colouration, morphometrics and ancient DNA

This appendix contains the publication of taxonomic research investigating the phenotypic and genetic variation in Pacific Robins in the Vanuatu Archipelago. I contributed to this work by performing the DNA extraction and sequencing of historical museum specimens. This article has been published in *Emu.*

Kearns A.M, **White, L.C**., Austin, J.J. & Omland K.E. (2015) Distinctiveness of Pacific Robin subspecies in Vanuatu revealed from disparate patterns of sexual dichromatism, plumage colouration, morphometrics and ancient DNA. *Emu* **115** 89-98.

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Distinctiveness of Pacific Robin subspecies in Vanuatu revealed from disparate patterns of sexual dichromatism, plumage colouration, morphometrics and ancient DNA

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Abstract. The Pacific Robin (*Petroica multicolor*) is a polytypic species, with 14 subspecies recognised in the tropical south-western Pacific Ocean. Like most of the avifauna of this region, the Pacific Robin has received little taxonomic attention since early works, such as those by Ernst Mayr in the 1930s–1950s. Here, we used mitochondrial DNA (mtDNA), spectrophotometry of plumage colouration and morphological data to examine patterns of sexual dichromatism, and phenotypic and genetic variation of Pacific Robins in the Vanuatu Archipelago. We have shown for the first time that subspecies in Vanuatu display three types of sexual dichromatism: typical marked sexual dichromatism, and reduced sexual dichromatism, in which both sexes have either elaborate masculinised plumage or drab feminised plumage. Different types of sexual dichromatism were not correlated with phylogenetic relationships. We also find that distinctive mtDNA, bill-length and colour of the throat plumage support the naming of a new subspecies for the population on Tanna Island, *P. m. tannensis* subsp. nov., which has long been treated as consubspecific with that on nearby Aneityum Island. Our study highlights the importance of revisiting the taxonomy and evolutionary history of other Pacific Ocean avifauna with molecular data and quantitative tests of phenotypic differentiation between island forms.

Additional keywords: insular speciation, island biogeography, mitochondria, Petroicidae, sexual dimorphism, taxonomy.

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Introduction

The Pacific Robin (*Petroica multicolor* Gmelin, 1789) is a textbook example of island speciation (Mayr 1963; Futuyma 1997) and was named as one of the 'great speciators' of the south-western Pacific Ocean by Mayr and Diamond (2001). The Pacific Robin is a small insectivorous passerine that is distributed across several archipelagos in the south-western Pacific: Solomon Islands (including Bougainville), Norfolk Island, Vanuatu, Fiji and Samoa (Fig. 1). Like several of the other species considered to be great speciators, such as the Golden Whistler complex (*Pachycephala pectoralis–melanura*) (Mayr 1942; Peterson 1996; Andersen *et al*. 2014), the Pacific Robin shows complex patterns of variation in sexual dichromatism across its range (Mayr 1934), ranging from marked sexual dichromatism (throat and dorsal plumage black in males and brown in females) to two different types of reduced sexual dichromatism, in which both sexes have either elaborate male-like plumage or drab female-like plumage (see Fig. S1 in the Supplementary Material available online only). Subspecies with marked sexual dichromatism, which is typical in Australo-Pacific *Petroica* robins, are found in every archipelago in the range of the Pacific Robin. However, subspecies in which both sexes have elaborate male-like plumage have only been described previously from Samoa and two of the Solomon Islands. In contrast, subspecies in which both sexes have drab female-like plumage are known only from some islands in Vanuatu (Mayr 1934).

Differences in the degree of sexual dichromatism, along with other minor variations in size and plumage characters, have been used to describe 14 allopatric subspecies: four in the Solomon Islands, one on Norfolk Island, three in Fiji, five in Vanuatu and one in Samoa (Mayr 1934, 1938; Boles 2007). Critically, past descriptions of subspecific limits were hindered by inadequate geographical series for some islands – particularly in Fiji, and the southern islands of Vanuatu – and by variation in sexually dichromatic plumage that made interpretation of age and sex difficult when series of specimens were small (e.g. differentiating between immature males, adult females, and adult males with female-like plumage in the case of drab plumage) (Mayr 1934).

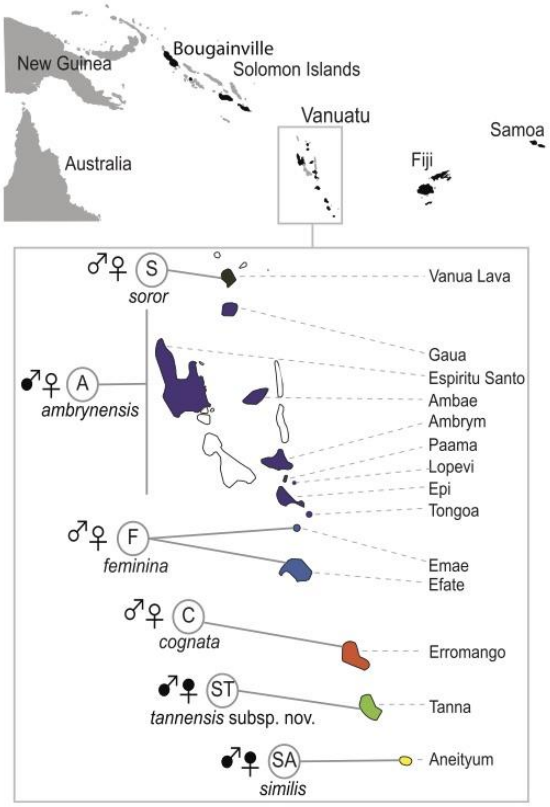

Fig. 1. Distribution of the Pacific Robin (Norfolk Island not shown), with details of subspecies and the geographical pattern of sexual dichromatism in the Vanuatu Archipelago. Filled male or female symbols indicate sex with elaborate male-like plumage and open male or female symbols indicate sex with drab female-like plumage. Only *P. m. ambrynensis* shows marked sexual dichromatism, in which males have elaborate plumage and females drab plumage. There are two types of reduced sexual dichromatism: either both sexes have elaborate male-like plumage (*P. m. tannensis* subsp. nov. of Tanna Island and *P. m. similis* of Aneityum Island) or both sexes have drab female-like plumage (subspecies *femina*, *cognata*, *soror*).

Molecular studies of similarly distributed, polytypic species, such as the Golden Whistler (Andersen *et al.* 2014) and the Variable Dwarf Kingfisher (*Ceyx lepidus*) (Andersen *et al.* 2013), have highlighted much incongruence between species boundaries in this region based on traditional, morphological taxonomy and those based on contemporary molecular techniques or studies. Recent molecular work (Christidis *et al.* 2011) suggests that the Pacific Robin could be sister to the more phenotypically divergent Red-capped Robin (*P. goodenovii*) of mainland Australia rather than the morphologically similar Scarlet Robin (*P. boodang*), with which it has often been considered conspecific (Schodde and Mason 1999). Critically, however, there has been little revision of taxonomic limits within the Pacific Robin since Ernst Mayr's seminal revision (Mayr 1934) based on specimens collected during the American Museum of Natural History (AMNH) Whitney South Sea Expeditions in 1921–41.

Here, we examine the patterns of sexual dichromatism and the distinctiveness of Pacific Robin subspecies in the Vanuatu Archipelago using mitochondrial DNA (mtDNA), plumage colour, length of bill and body size. Of particular interest is the distinctiveness of forms on the two southernmost islands of Vanuatu: Tanna and Aneityum (Fig. 1). In his revision of the Pacific Robin, Mayr (1934) examined only a small series from these two islands: two adult males, four immature males and one immature female from Aneityum, and one adult male and one immature male from Tanna. Based on his small sample, Mayr (1934) provisionally referred birds from Tanna and Aneityum to *P. m. similis* Gray, 1859, along with birds from nearby Erromango Island (Fig. 1). Birds on these three southern islands were thought to display marked sexual dichromatism, as shown by *P. m. ambrynensis* of the central Vanuatu Archipelago (see Fig. 1). Upon examination of the additional specimens collected by L. Macmillan between 1935 and 1937, birds from Erromango were found to have males with drab female-like plumage like the northern and central subspecies *P. m. soror* and *P. m. feminina* (Fig. 1), and were thus described as a new subspecies, *cognata* (Mayr 1938). However, to our knowledge, there has been no published update of the characteristics of the populations of Aneityum and Tanna Islands based on this expanded series collected by Macmillan (Mayr 1938).

Methods

Phenotypic sampling

We took measurements – morphometric and spectrophotometric – of 78 adult birds held at the AMNH, New York, and one adult female from the Florida Museum of Natural History in Gainesville (for details of specimens for each subspecies, see Table S1 in the Supplementary Material). We had five specimens of *P. m. soror* (4 males, 1 females), 23 *P. m. ambrynensis* (14 males, 9 females), 11 *P. m. feminina* (5 males, 6 females), 17 *P. m. cognata* (10 males, 7 females) and 23 *P. m. similis* (14 males and 9 females). Of the 23 *similis*, seven specimens (4 males, 3 females) were from Aneityum and 16 specimens (10 males, 6 females) from Tanna. Note that owing to damage and wear, not all specimens were measured for each morphometric variable and plumage patch. Furthermore, sample sizes of female *soror* were insufficient for reliable pairwise comparisons.

Morphometrics

We measured length of wing (chord flattened from the carpal joint to the tip of longest primary feather) and tail (length of longest rectrix from tip to the point where it emerged from the body) using a stopped ruler, to the nearest 1 mm. Bill-length was measured from the middle of the nostril to the tip of the bill, to the nearest 0.1 mm, using digital calipers (Mitutoyo CD-6" CX, Takatsu-ku, Kawasaki, Kanagawa, Japan). Two-tailed *t*-test showed that males and females from Tanna and of *P. m. ambrynensis* were sexually dimorphic for wing-length (Tanna: $t = 3.81$, d.f. = 14.0, $P = 0.002$; *ambrynensis*: $t = 2.41$, d.f. = 12.7, $P = 0.032$), whereas only *ambrynensis* showed sexually dimorphism in bill-length ($t = 2.18$, d.f. = 12.2, $P = 0.05$) (Table 1). Thus, morphometric variables of males and females were

Table 1. Morphometrics of Pacific Robins from Vanuatu and *t*-tests of sexual dimorphism of each variable for each taxon
Significant results of *t*-tests are highlighted in bold (with degrees of freedom as subscripts)

	Sex	*N*	Wing-length Mean	Range	s.e.	*t*-test	*N*	Tail-length Mean	Range	s.e.	*t*-test	*N*	Bill-length Mean	Range	s.e.	*t*-test
soror	F	1	62	62–62	–	–	1	40	40–40	–	–	1	8.7	8.7–8.7	–	–
	M	4	65	63–66	0.71		4	41	39–42	0.63		4	8.6	8.2–8.7	0.14	
ambrynensis	F	9	61	56–65	0.94	$\mathbf{t_{12,7}=2.41, P=0.032}$	9	39	33–43	0.87	$t_{13,1}=0.08, P=0.94$	7	8.1	7.7–8.5	0.10	$\mathbf{t_{12,2}=2.18, P=0.05}$
	M	14	63	60–67	0.51		14	39	37–43	0.49		14	8.4	8.0–8.9	0.07	
feminina	F	6	64	62–68	1.0	$t_{8,1}=1.39, P=0.20$	6	41	37–44	1.01	$t_{7,9}=1.46, P=0.18$	6	8.3	8.0–8.6	0.07	$t_{6,2}=1.98, P=0.09$
	M	5	66	63–70	1.26		5	43	42–45	0.6		5	8.6	8.2–9	0.13	
cognata	F	7	62	60–64	0.58	$t_{13.5}=1.63, P=0.13$	7	43	39–45	0.97	$t_{9,9}=0.99, P=0.34$	6	8.5	8.1–8.8	0.12	$t_{10,4}=0.56, P=0.58$
	M	10	63	60–65	0.51		10	44	41–47	0.55		10	8.6	8.3–9.2	0.09	
Aneityum	F	3	62	62–63	0.44	$t_{4.93}=2.18, P=0.08$	3	44	43–45	0.44	$t_{3,6}=1.17, P=0.31$	3	8.6	8.4–8.7	0.08	$t_{4,7}=1.90, P=0.12$
	M	4	64	63–65	0.48		4	45	42–48	1.39		4	8.8	8.7–9	0.07	
Tanna	F	6	60	59–63	0.57	$\mathbf{t_{14,0}=3.81, P=0.002}$	6	44	43–46	0.49	$t_{14,0}=0.57, P=0.58$	5	8.2	7.9–8.6	0.11	$t_{8,8}=1.33, P=0.22$
	M	10	64	60–68	0.74		10	44	42–48	0.69		9	8.4	8.0–8.8	0.09	

analysed separately. Analysis of variance (ANOVA) with Tukey–Kramer honestly significant difference (HSD) test for *post hoc* pairwise comparisons of means were used to test for differentiation across all six forms in Vanuatu (*soror*, *ambrynensis*, *feminina*, *cognata*, Tanna Island, Aneityum Island). Two-tailed *t*-tests assuming uneven variances were used to test specifically for differentiation of Tanna and Aneityum. All statistics were performed in JMP 10 (SAS Inc., Cary, NC).

Spectrophotometry

We measured the reflectance of the plumage of the back, crown, throat and breast tract using an Ocean Optics USB2000 reflectance spectrophotometer with a PX-2 pulsed xenon light source (Ocean Optics, Dunedin, FA, USA). Measurements were calibrated with a white Spectralon standard (Labsphere, North Sutton, NH, USA) for the first measurement of the sitting and either when the curve of the white standard deviated from normal (this was checked between each plumage tract) or after a maximum of five birds were measured. Three measurements were taken for each plumage tract for each individual using an integration time of 20 ms, and spectra average of 40 with a multiple strobe setting and a constant distance and angle from the bird. The raw spectra files were imported and processed into 5 nm bins within the avian visual spectrum (wavelength of 320–700 nm) using a macro for Microsoft Excel 2010 (M. Double unpubl. data; Microsoft Corporation, Redmond, WA, USA). Mis-measurements were screened visually using line plots for each patch per individual in Excel.

We used JMP 10 to calculate the mean reflectance curve for each plumage tract for each individual and also the mean of all males and females from Tanna and Aneityum Islands. CLR v1.05 (Montgomerie 2008) was used to calculate tristimulus colour variables from the mean reflectance curves (Hill and McGraw 2006) using formula B1 (R320–700) for 'brightness', formula S5a ($S_5 = \sqrt{(B_r - B_g)^2 + (B_y - B_b)^2}$) for 'saturation/chroma' and formula H4a (arctan {[(By − Bb)/B1] / [(Br − Bg)/B1]}, where b (blue) = 400–475 nm, g (green) = 475–550 nm, y (yellow) = 550–625 nm, and r (red) = 625–700 nm)) for 'hue'.

We first used spectrophotometry of the plumage of the back, crown and throat to explore quantitatively the different patterns of sexual dichromatism in Vanuatu. We then tested for differences in brightness, hue and saturation of each plumage tract between subspecies and island forms that had the same type of pattern of sexual dichromatism. We used ANOVA with Tukey–Kramer HSDs for *post hoc* pairwise comparisons of means to test for differentiation of the three drab-plumaged forms (*soror*, *feminina*, *cognata*), and a two-tailed *t*-test assuming unequal variances for elaborately plumaged forms from Tanna and Aneityum Islands. Only *ambrynensis* had sexually dichromatic plumage, so no comparisons were calculated within this plumage-type. Our *a priori* prediction is that the different plumage tracts and morphometric variables could each show independent differences. We therefore do not correct for multiple comparisons across different plumage tracts and morphometric measurements. However, values of *P* from non-independent tests of the tristimulus colour variables (hue, saturation, brightness) within each plumage tract were adjusted using Bonferroni correction ($\alpha = 0.05/3 = 0.0167$).

Molecular sampling

MtDNA *ND2* was sequenced from three individuals from both Aneityum and Tanna Islands, and also from the four other subspecies in Vanuatu (see Table S1 for details of samples). Three cryo-frozen tissues were available from *P. m. ambrynensis* from Espiritu Santo. For the rest, we sampled the toe-pad from museum specimens at the AMNH. All sequences were deposited in GenBank (accession numbers KP203816–KP203833).

Extraction and sequencing: contemporary tissue samples

DNA was extracted from frozen tissues using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). *ND2* was amplified from DNA extracted from frozen tissues using L5215 (TATCGGGCCCATACCCCGAAAAT; Hackett 1996) and H6313 (ACTCTTRTTTAAGGCTTTGAAGGC; Sorenson *et al.* 1999). PCR used 2 µL 10× Gold buffer, 1.25 µL MgCl, 1.2 µL dNTP (10 µM), 0.4 µL each primer (10 µM), 0.15µL AmpliTaq Gold (all Applied Biosystems, Roche, NJ, USA), 1.2 µL DNA template and 8.4 µL H_20, and the following thermocycle conditions: 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and then a final extension step of 72°C for 3 min. PCR products were cleaned using ExoSapIT and then sequenced commercially (Genewiz Inc., South Plainfield, NJ, USA). Sequencer 4.7 (Gene Codes, Ann Arbor, MI) was used to edit and align sequences, and to check for stop codons.

Extraction and sequencing: historical museum samples

We controlled for contamination of historical museum samples with contemporary DNA and previously amplified mtDNA PCR products of the ND2 gene by conducting all work preliminary to PCR in a dedicated, physically separate, cleanroom facility at the Australian Centre for Ancient DNA, University of Adelaide. No contemporary avian samples or DNA had ever been present in the pre-PCR laboratory. The laboratory included the use of dead-air glove boxes fitted with internal ultraviolet (UV) lights for extraction of DNA and set-up of PCR; regular decontamination of all work areas and equipment with sodium hypochlorite; personal protective equipment, including disposable laboratory gowns, face masks, face shields, shoe covers and double-gloving; and strict one-way movement of personnel.

Sampling restrictions and methodology aimed at minimising destructive sampling of museum specimens limited toe-pad sample size to enough for just a single DNA extraction attempt for all historical museum skins. DNA was extracted using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions with the following modifications: (1) 12uL of 1M DTT was added to each sample before proteinase K digestion; (2) 2 µL of carrier RNA was added to each sample following the addition of ethanol; and (3) DNA was eluted twice from the silica spin columns with 50 µL of buffer AE for a final volume of 100 µL. A negative extraction control was included to monitor for contamination.

The mtDNA ND2 gene was amplified using seven sets of primers targeting ~200–260 base pairs (bp) overlapping fragments. Primer combinations were: fragment 1 (L5216/5′-GGG GGTGGTGGGATTTTGAG-3′), fragment 2 (5′-GAAATCAA TACACTRGCCATC-3′/5′-AAAAGTGAAATGGAACCAGT C-3′), fragment 3 (5′-ATAGTCAACGCATGATCCAC-3′/5′-AGAGGATGGCTATAGTGGTT-3′), fragment 4 (5′-CCTC CCACTCACTARACACCA-3′/5′-AGGAAYRCGGCTGCGG TTAT-3′), fragment 5 (5′-GCCATYATCATCGCCTACAACC-3′/5′-TGAATGATAAGTCATTTYGGGAGGA-3′), fragment 6 (5′-CTACTCTCCCTAGCAGGCCT-3′/5′-ATGTGGTTGGTG GTGTGGG-3′), and fragment 7 (5′-CCCTGCTAGGGCTATT CTTCT-3′/H6313). PCRs were performed in 25 µL volumes containing 2 µL of extracted DNA, 1 × High Fidelity PCR Buffer (Invitrogen, Carlsbad, CA, USA), 200 µM of each dNTP, 400 µM of each primer (Geneworks, Hindmarsh, SA, Australia), 2 mM $MgSO_4$, 1 µg $µL^{-1}$ RSA (Sigma, St Louis, MO, USA) and 0.5 units of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). Thermocycling conditions were: denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 30 s, with a final extension step at 68°C for 10 min. All PCR attempts included a PCR no-template control and the relevant negative extraction control or controls for the samples being amplified.

PCR products were visualised under UV light on 2–3.5% agarose gels stained with ethidium bromide. Successful amplifications were sent to the Australian Genome Research Facility (Adelaide, SA) for Sanger Sequencing and capillary electrophoresis. Sequence chromatograms were edited and assembled using Geneious 7.1.2 (http://www.geneious.com, Kearse *et al.* 2012).

Phylogenetic analysis

We used TCS 1.2 (Clement *et al.* 2000) to calculate haplotype networks using a 95% connection limit, and DnaSP 5.10 (Rozas *et al.* 2003) to measure the net divergence between subspecies and specimens from Aneityum and Tanna Islands using the *Dxy* statistic. Owing to small sample sizes we did not explore nucleotide or haplotype diversity statistics.

Results

Differences in sexual dichromatism across Vanuatu

Quantitative analysis of plumage colouration differentiated three types of sexual dichromatism in Vanuatu (Fig. 2, Fig. S1): (1) marked sexual dichromatism (*ambrynensis*); (2) reduced dichromatism with drab female-like plumage (*soror*, *feminina*, *cognata*); and (3) reduced dichromatism with elaborate male-like plumage (populations on Tanna and Aneityum Islands). This is the first time elaborate reduced dichromatism has been documented in the Vanuatu Archipelago (Mayr 1934, 1938). Elaborate females from Tanna and Aneityum Islands have dark sooty black dorsal plumage and throats that approach those of males of those islands (Fig. S2), as well as males from sexually dichromatic subspecies such as *ambrynensis* (Fig. 2, Fig. S1).

The three plumage types were clearly differentiated from each other in spectrophotometry of the back, crown and throat plumage (Figs 2, 3). Males with black plumage (dichromatic and elaborate types) and brown plumage (drab type) differed in the brightness, saturation and hue of the plumage of the back, crown and throat (Fig. 3; Table S2; ANOVA: F 30.07–119.03, d.f. 2, 35–38, $P < 0.0001$). Brightness and hue of back plumage discretely separated subspecies with black (dichromatic and elaborate)

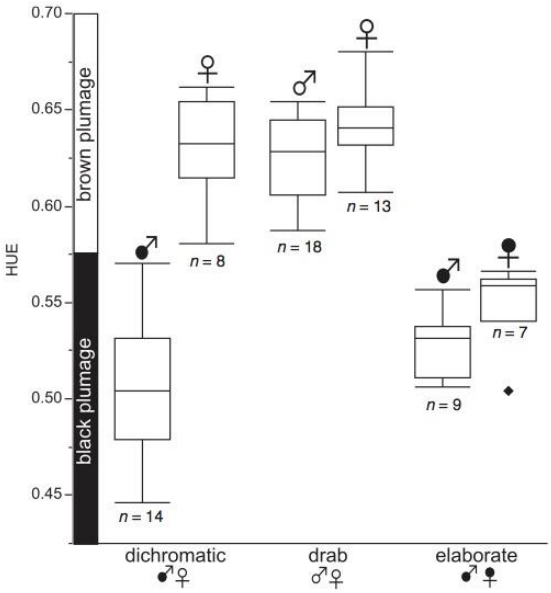

Fig. 2. Vanuatu robins show three distinct types of sexual dichromatism: (1) marked dichromatic where males have elaborate black plumage and females have drab brown plumage (*ambrynensis*); (2) reduced drab dichromatic where both sexes have drab female-like plumage (*soror*, *feminina*, *cognata*); and (3) reduced elaborate dichromatic where both sexes have elaborate male-like plumage (populations on Tanna and Aneityum Islands). See Fig. S1 for photos of each plumage type. The three plumage-types showed significant differences in back, crown and throat plumage in both sexes (Table S2). Box-plots show variation (minimum, 25% quantile, median, 75% quantile and maximum) in the hue tristimulus colour variable (H4a) for the back plumage patch across the three plumage-types for each sex. The filled diamond represents an outlier data point. Sample sizes for each plumage-type and sex are shown below each box-plot. Filled and unfilled sex symbols indicate the plumage type of each sex (filled, elaborate; unfilled, drab).

and brown (drab) plumage (Fig. 3). However, crown and throat plumage showed more of a continuous gradient in these variables from pure black in *ambrynensis* through sooty black on Tanna Island to grey on Aneityum Island to brown in subspecies *soror*, *cognata* and *feminina* (Fig. 3). A similar, but weaker, trend is seen in females, differentiating subspecies with sooty black (elaborate type) and brown (drab and dichromatic types) dorsal plumage (Fig. 3). However, in females the only statistical differences after Bonferroni correction ($\alpha = 0.017$) were in hue and saturation of the back and crown and saturation of the throat (Table S2; ANOVA: F14.94–42.99, d.f. 2, 23–26, $P < 0.0001$). Female dorsal and throat plumage showed no significant differences in brightness between the three plumage types (Table S2; ANOVA: F1.53–4.7, d.f. 2, 23–26, $P = 0.02$–0.24).

Differences among subspecies with drab reduced sexual dichromatism

Quantitative analyses revealed only minor differences among the three subspecies with drab female-like plumage (*soror*, *feminina*, *cognata*) in either morphometric variables or plumage colouration (Table S3). Brightness, saturation and hue variables of the three drab subspecies overlapped substantially in all four plumage tracts (e.g. brightness and hue shown in Fig. 3). Only saturation of breast plumage in females (ANOVA: F 10.8, d.f. 2,10, $P = 0.003$) and breast hue in males (ANOVA: F 7.4, d.f. 2,16, $P = 0.005$) were found to be significantly different across all three subspecies after Bonferroni correction ($\alpha = 0.017$). However, this result should be treated with caution as it appeared to be based solely on differences between poorly sampled *soror* versus *feminina* and *cognata* (Table S3). No other differences in plumage colouration were detected (Table S3). Spectrophotometry thus does not strongly support the description of Mayr (1934) that *soror* has darker upperparts than *feminina* and *cognata*. Wing-length and tail-length differed significantly between males of the drab subspecies (ANOVA: wing: F 6.3, d.f. 2,16, $P = 0.01$; tail: F 5.2, d.f. 2,16, $P = 0.02$), but not females (ANOVA: wing: F 2.9, d.f. 2,11, $P = 0.1$; tail: F 1, d.f. 2,11, $P = 0.38$) (Table S3). Bill-length showed no differences between the three drab subspecies in either sex (Table S3). *Post hoc* pairwise comparisons showed significant differences in tail-length between male *cognata* and *soror* (Tukey's HSD: $P = 0.014$), and wing-length of male *feminina* and *cognata* (Tukey's HSD: $P = 0.0093$).

Differences among subspecies with elaborate reduced sexual dichromatism

We were able to examine a more complete geographical series than that examined by Mayr (1934), consisting of 10 adult males and six adult females from Tanna and four adult males and three adult females (cf. Mayr's one adult male from Tanna and two adult males from Aneityum). The biggest difference in colouration between the islands was that birds from Tanna had darker throats than those from Aneityum in both sexes (Fig. 4, Fig. S2). On Tanna, males had black throats and females mid-grey throats, whereas on Aneityum, males had dark slate-grey throats and females white throats patchily mixed with grey (Fig. 4, Fig. S2). Throat plumage of males from Aneityum and Tanna differed statistically in brightness ($t = -4.60$, d.f. $= 5.0$, $P = 0.006$) and saturation ($t = -3.80$, d.f. $= 5.0$, $P = 0.013$), but not hue ($t = -1.40$, d.f. $= 5.0$, $P = 0.22$) (Table S4). Males also differed in breast plumage, with males from Tanna being darker red than males from Aneityum ($t = -3.9$, d.f. $= 4.8$, $P = 0.013$; Fig. 4). No statistically significant differences in plumage colour were found in females (Table S4), despite throat plumage being visibly different to human observers and in the mean reflectance spectra (Fig. 4, Fig. S2). Bill-lengths from Aneityum were longer than those from Tanna in both sexes (males: $t = -3.49$, d.f. $= 10.1$, $P = 0.006$; females: $t = -2.82$, d.f. $= 6$, $P = 0.03$; Table 1, Table S4). There were no differences between Aneityum and Tanna in wing-length or tail-length in males; females showed differences in wing-length ($t = -2.77$, d.f. $= 6.8$, $P = 0.03$) but not tail length (Table S4).

Broad morphometric patterns

Length of wing and tail differed across all subspecies and island forms in Vanuatu in both sexes (Table 1; ANOVA: Wing-length – female: $F_{5,26} = 2.57$, $P = 0.05$; male: $F_{5,41} = 2.82$, $P = 0.028$; Tail-length – female: $F_{5,26} = 3.74$, $P = 0.011$; male: $F_{5,41} = 11.82$,

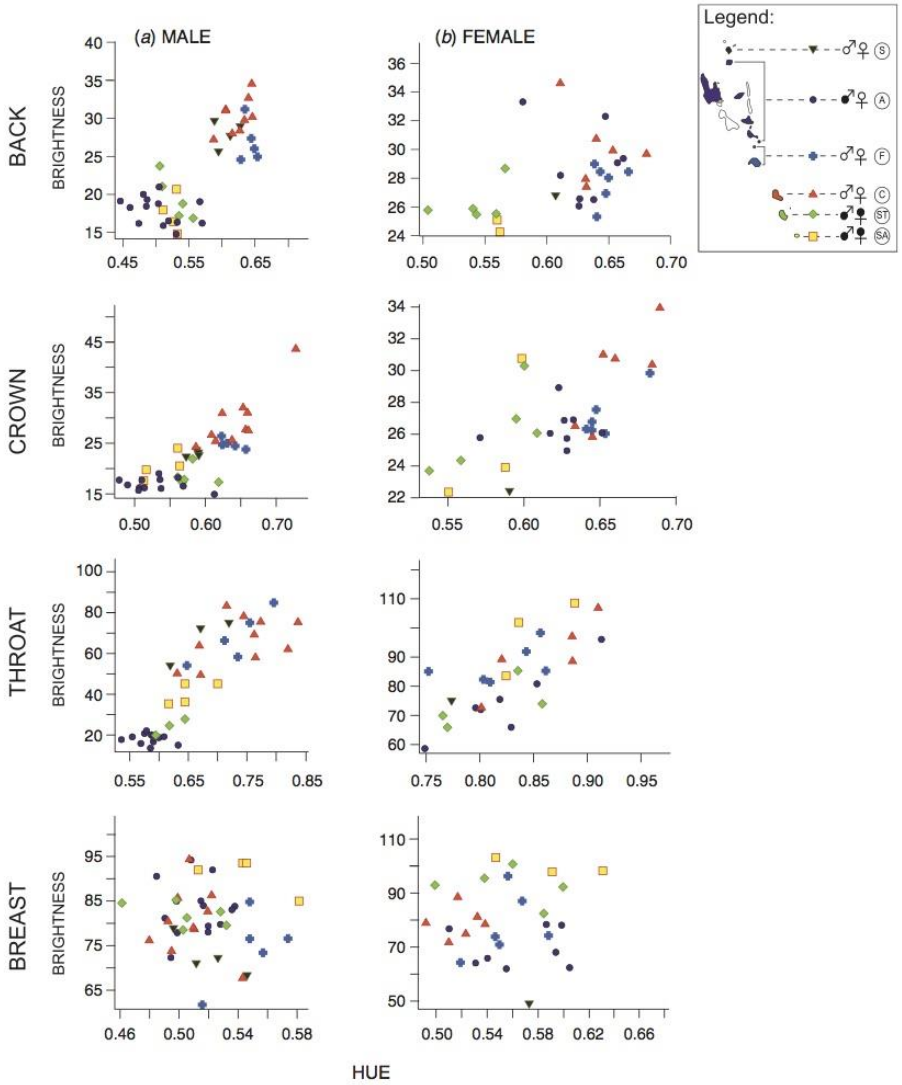

Fig. 3. Variation in brightness (B1) and hue (H4a) tristimulus colour variables for four plumage tracts in (*a*) male and (*b*) female Pacific Robins in Vanuatu. Legend shows islands of Vanuatu Archipelago with colour-coded symbols used in the plots, and with letters in circles identifying subspecies or island forms following Fig. 1: S, *soror*; A, *ambrynensis*; F, *feminina*; C, *cognata*; ST, *tannensis* subsp. nov., from Tanna Island; SA, *similis*, from Aneityum Island. Filled and unfilled sex symbols indicate the plumage type of each sex (filled, elaborate; unfilled, drab) and thus denote whether subspecies show dichromatic, drab or elaborate forms of sexual dichromatism.

$P < 0.001$). However, only females differed in bill length across Vanuatu (ANOVA: female: $F_{5,22} = 3.12$, $P = 0.028$; male: $F_{5,40} = 2.25$, $P = 0.068$). *Post hoc* pairwise comparisons of means were borderline significant for longer-winged females of *feminina* compared with *ambrynensis* and Tanna females (Tukey's HSD: $P < 0.05$), and also for male *ambrynensis* compared with *cognata*, which had similar wing-lengths (Tukey's HSD: $P < 0.04$) (Table 1). Pairwise comparisons showed that male *ambrynensis* had shorter tails than all other subspecies (Tukey's HSD: all $P < 0.02$), but that female *ambrynensis* only differed from females from Tanna Island (Tukey's HSD: $P = 0.02$). All other *post hoc* comparisons were non-significant at $\alpha = 0.05$.

MtDNA phylogeography

We obtained 940 bp of ND2 sequence for all individuals except one individual from Tanna (AMNH-336844), which we

sequenced 905 bp. We analysed two datasets. One contained the full 940 bp sequenced for 17 individuals and the other contained 905 bp sequenced for all 18 individuals. The 940 bp dataset showed no sharing of *ND2* haplotypes among the subspecies, nor among birds from Aneityum ($n=3$) and Tanna ($n=2$) (Fig. 5), which were 0.32% divergent (*Dxy*) (Table 2). All haplotypes radiated from a single internal haplotype sampled from one *feminina* individual from Efate Island (AMNH-212428). The other haplotype belonging to *feminina* was sampled from the two individuals from Emae Island. The most divergent subspecies was *soror*, which is found on the northernmost island in the archipelago (*Dxy* 0.5–0.64%; Table 2).

The haplotype network calculated from the 905 bp dataset ($n=18$) differed from the 940 bp dataset in two respects. First, the individual from Tanna (AMNH-336844) not in the 940 bp dataset had a unique haplotype that was 2 bp divergent from the haplotype sampled in the other two birds from Tanna (AMNH-336842, AMNH-336857) (Fig. 5). Second, because the 1 bp mutation that differentiated *ambrynensis* from the internal *feminina* haplotype was not present, the internal haplotype in the

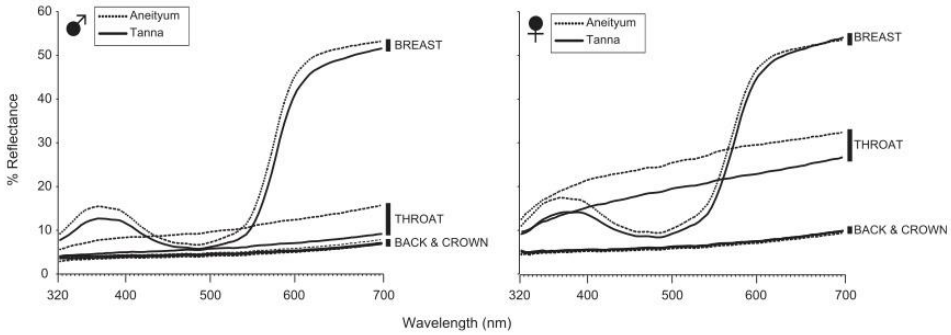

Fig. 4. Mean reflectance spectra for the plumage of the back, crown, throat and breast of populations from Aneityum Island (dashed line; $n=4$ males, 3 females) and Tanna Island (solid line; $n=10$ males, 6 females). Note the difference in reflectance of the plumage of the throat between Tanna and Aneityum in both sexes. See Fig. S2 for photos of skins from Aneityum and Tanna Islands.

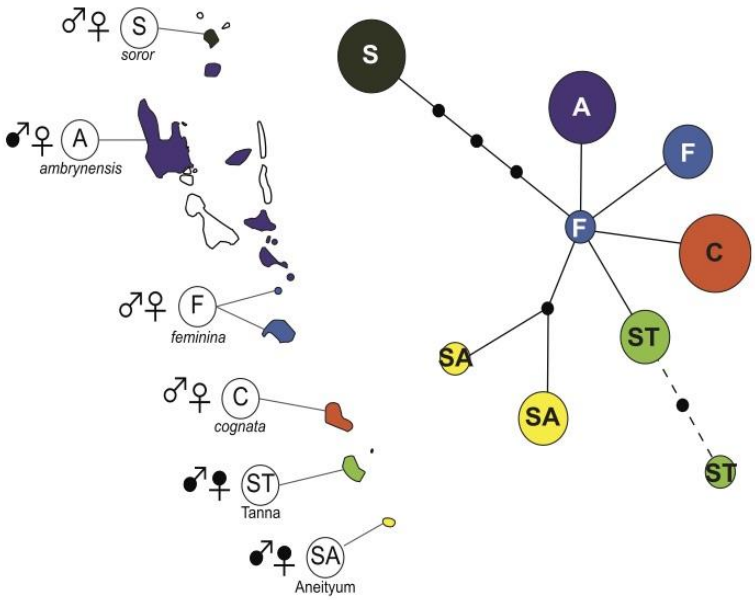

Fig. 5. Unrooted haplotype network of Pacific Robins in Vanuatu inferred from 940 bp of mitochondrial *ND2*. Haplotypes are coloured-coded and labelled (letters within circles) by subspecies or island form (see caption to Fig. 3 for details). Small black circles represent unsampled haplotypes. The dashed line shows the inferred position of a second *ND2* haplotype from Tanna Island based on a shorter 905 bp dataset available for all 18 sampled individuals.

Table 2. Net mtDNA *ND2* divergence (*Dxy*) of Pacific Robin subspecies and island forms in Vanuatu

Dxy is expressed as a percentage, e.g. $Dxy = 0.0053 \times 100 = 0.53\%$

	soror	*ambrynensis*	*feminina*	*cognata*	Tanna	Aneityum
soror						
ambrynensis	0.53					
feminina	0.50	0.18				
cognata	0.53	0.21	0.18			
Tanna	0.53	0.21	0.18	0.21		
Aneityum	0.64	0.32	0.28	0.32	0.32	

905 bp network contained three *ambrynensis* and one *feminina* individuals. Importantly, in both the 905 bp and 940 bp datasets there are a minimum of 3 bp differences that separate all sampled individuals from Tanna and Aneityum Islands (Fig. 5).

Discussion

The Pacific Robin shows substantial diversity in plumage, size and patterns of sexual dichromatism across its range in the south-western Pacific Ocean (Mayr 1934; Schodde and Mason 1999; Boles 2007). However, the species has received little taxonomic attention since the revision of Mayr (1934). In this study, we explored patterns of sexual dichromatism and tested the taxonomic limits of the five named subspecies in the Vanuatu Archipelago using mtDNA and quantitative measures of phenotypic differentiation. We found support for the phenotypic and genetic distinctiveness of six forms that ought to be recognised as subspecies. We also show that three types of sexual dichromatism are shown by subspecies in Vanuatu, and that these are not associated with phylogenetic relationships.

Taxonomic limits of Pacific Robins in Vanuatu

Our data support the recognition of six genetically or phenotypically distinct forms in Vanuatu, which includes four described subspecies (*soror*, *ambrynensis*, *cognata*, *feminina*) and the currently consubspecific forms on Tanna and Aneityum Islands (currently treated as *P. m. similis*). The weakest support is for the distinctiveness of *cognata* and *feminina*, both of which have drab reduced dichromatism. Our examination found little evidence of different plumage colouration between the subspecies (Table S3) and the two subspecies are weakly genetically divergent compared with most other subspecies (Dxy 0.18%; Fig. 5). Notably, however, drab *feminina* and dichromatic *ambrynensis* were also only weakly genetically divergent (Dxy 0.18%) despite having different types of sexual dichromatism. In contrast, *soror* compared with *cognata* and *feminina* showed marked genetic divergence (Dxy 0.50–0.53%; Fig. 5) despite being largely indistinguishable by phenotype (Fig. 3). This is consistent with a conclusion that plumage is a poor indicator of phylogenetic relationships in Pacific Robins, a phenomenon observed in other Pacific species, such as the Golden Whistler (Andersen *et al.* 2014).

We found strong support for the distinctiveness of the populations of Tanna and Aneityum Islands on the basis of divergence of mtDNA haplotypes, plumage colouration and bill-length. The level of phenotypic and genetic differentiation is comparable to that of other described subspecies in Vanuatu. Indeed, both human visual inspection and quantitative tests of plumage colouration show that Aneityum and Tanna are more phenotypically distinct from one another (Fig. 4, Fig. S2) than the three subspecies with the drab form of sexual dichromatism are to each other (*cognata*, *feminina*, *soror*). Populations of Aneityum and Tanna Islands also have a similar degree of genetic distinctiveness to that shown by the other subspecies in Vanuatu (e.g. Dxy of 0.32% between Tanna and Aneityum versus Dxy of 0.21% between *cognata* and *ambrynensis*; Table 2).

As is typical of studies of Pacific Island birds, our sample size was constrained by the remoteness of the region and the large range inhabited by Pacific Robins. Few specimens of robins from Aneityum and Tanna Islands have been collected. Most of the specimens examined in this study were not available to Mayr (1934, 1938) and there have been no subsequent studies of robins in Vanuatu. Similarly small sample sizes were used by Mayr (1934, 1938) in his seminal taxonomic revision of Pacific Robins, as well as to describe countless other avian species and subspecies from the Pacific region. Although additional fresh tissues would enable more robust taxonomic conclusions and exploration of evolutionary history, we believe that our sampling is sufficient to achieve our goal to revise the taxonomy of Pacific Robins in Vanuatu based on measures of both genetic and phenotypic distinctiveness.

We propose that populations on Tanna and Aneityum Islands should be described as two distinct subspecies. Our data suggest that the observed phenotypic and genetic variation of Aneityum and Tanna Islands is geographically discrete and representative of each population rather than the result of individual variation. The advantages and disadvantages of naming such distinct island forms as subspecies has been debated extensively elsewhere (e.g. de Queiroz 2005; Patten 2010; Winker 2010; Haig and Winker 2010; Gill 2014). Here, we note that lumping these phenotypically and genetically distinct forms into a single monotypic species or subspecies would fail to capture the observed biodiversity of Pacific Robins in Vanuatu. This in turn could have serious repercussions for any future conservation management decisions for these populations that are increasingly at risk from habitat loss and climate change.

Formal description of new subspecies

Petroica multicolor similis Gray, 1859, was first described from birds from Aneityum Island. Thus, here, we describe a new subspecies for the population restricted to Tanna Island: *Petroica multicolor tannensis* Kearns & Omland subsp. nov. Holotype: American Museum of Natural History (AMNH), No. 336842, male, Tanna Island, Vanuatu, L. Macmillan, 1936.

Diagnosis

Petroica multicolor tannensis subsp. nov. and *P. m. similis* of Aneityum Island differ from sexually dichromatic *P. m. ambrynensis* and drab, female-like *P. m. cognata*, *P. m. feminina* and *P. m. soror* in exhibiting reduced sexual dichromatism, in which both males and females have male-like dull, sooty black dorsal plumage. Males of *P. m. tannensis* subsp. nov. differ from those of *P. m. similis* in having a darker black throat and more saturated red on the breast. Adult females of *P. m. tannensis* subsp. nov. differ from those of *P. m. similis* in having a darker grey throat. In contrast, males of *P. m. similis* have slate-grey

throats and less-saturated orange-red breasts, and females have predominately white throats with patches of grey. The bill of *P. m. tannensis* subsp. nov. is shorter than that of *P. m. similis* in both sexes (*tannensis*: males 8.0–8.8 mm, females 7.9–8.6 mm; *similis*: males 8.7–9.0 mm, females 8.4–8.7 mm). *ND2* sequences of *P. m. tannensis* were not shared with any other subspecies, and sequence divergence (*Dxy* statistic) was 0.32% from *P. m. similis* of Aneityum Island and 0.18–0.53% from other Vanuatu subspecies of Pacific Robin.

Distribution

Petroica multicolor tannensis subsp. nov. is endemic to Tanna Island in the Vanuatu Archipelago.

Etymology

tannensis refers to the endemicity of this subspecies to Tanna Island.

Is sexual dichromatism correlated with phylogeny?

Many researchers have compared levels of dichromatism among closely related species and subspecies, including the phenomenon of island forms that are less sexually dichromatic than their mainland congeners (Mayr 1942; Peterson 1996; Badyaev and Hill 2003). Typically this involves the reduction of elaborate plumage ornamentation in males to approach the more drab or cryptic female plumage. However, sexually monochromatic island forms can also result from an increase in elaborate plumage ornamentation in females (Badyaev and Hill 2003; Friedman *et al.* 2009). In this study, we showed for the first time that Pacific Robins in Vanuatu exhibit three types of sexual dichromatism. Marked sexual dichromatism is only evident in *ambrynensis*, whereas the other subspecies of the archipelago have reduced sexual dichromatism in which either both sexes have drab female-like plumage (*soror*, *feminina*, *cognata*) or both sexes have elaborate male-like plumage (*P. m. tannensis* subsp. nov. of Tanna Island and *P. m. similis* of Aneityum Island).

Critically, neither form of reduced sexual dichromatism is truly sexually monochromatic. Males remain more elaborate than females in all cases in Vanuatu (Fig. 3, Fig. S1). Although not analysed quantitatively, it appears that this pattern holds for the other three Pacific Robin subspecies with elaborate reduced dichromatism: female *P. m. pusilla* of Samoa are described as having greyer throats than males, and female *P. m. dennisi* and *P. m. polymorpha* in the Solomon Islands have either brown or russet heads and lighter throats than their black-headed males (Mayr 1934).

Unlike in the Eastern Bluebird in Bermuda (*Sialia sialis bermudensis*) (Avery*et al.* 2014), reduced sexual dichromatism in Pacific Robins does not seem to be associated with changes in both sexes (Fig. 2), that is females of drab-plumaged *cognata* and *feminina* do not differ substantially in plumage from females of dichromatic *ambrynensis*, nor does there appear to be a difference between males of elaborately plumaged *P. m. dennisi* in the Solomon Islands compared with males of typically dichromatic *ambrynensis* (Mayr 1934). One possible exception to this is the populations on Tanna and Aneityum Islands, which have lighter sooty black upperparts compared with the deep-black plumage described for the other elaborately plumaged birds from Samoa and the Solomon Islands (Mayr 1934), as well as males of typically dichromatic *ambrynensis* (Fig. 2). Testing these dynamics quantitatively was beyond the geographical scope of this study, but would undoubtedly be illuminating if quantitative phenotypic analyses were extended across the complete range of the Pacific Robin.

Although geographically limited to Vanuatu, our data are able to dismiss one major hypothesis about the evolution of reduced sexual dichromatism in Pacific Robins: that differing patterns of sexual dichromatism are determined by phylogeny. Instead, we found that subspecies with the same plumage-type are not more closely related to each other than to subspecies with other plumage types (Fig. 5, Table 2). Thus, the different occurrences of each type of sexual dichromatism is likely a result of convergence, perhaps owing to similar sexual or natural selective forces on the different islands in which they appear, or simply owing to random genetic drift on different islands (Grant 2001).

An integrative approach using genomics, behaviour and quantitative tests of phenotypic variation across all Pacific Robins would be necessary to tease apart the factors that have caused the evolution and maintenance of this geographical mosaic of dichromatism types. Such a study would not only add to our understanding of the common evolutionary drivers of sexual ornamentation in birds, but would also contribute to our knowledge of the common evolutionary processes that have shaped the history of biota in the understudied tropical south-western Pacific Ocean (Filardi and Moyle 2005; Kirchman and Franklin 2007; Phillimore *et al.* 2008; Moyle *et al.* 2009; Uy *et al.* 2009; Clegg and Phillimore 2010; Andersen *et al.* 2013, 2014; Irestedt *et al.* 2013; Jønsson *et al.* 2014). Most importantly, such a study would offer a framework for a comprehensive taxonomic revision of Pacific Robins, which is needed to ensure the recognition and effective management of threatened populations, such as the endangered *P. m. multicolor* on Norfolk Island (Garnett *et al.* 2011; Department of National Parks 2010).

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Appendix III

Norfolk Island Robins are a distinct endangered species: Ancient DNA unlocks surprising relationships and phenotypic discordance within the Australo-Pacific Robins

This appendix contains the publication of research investigating the phylogenetic relationship of the Norfolk Island Robins to other Pacific Robin species.. I contributed to this work by performing the DNA extraction and sequencing of historical museum specimens. This article has been published in *Conservation Genetics.*

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RESEARCH ARTICLE

Norfolk Island Robins are a distinct endangered species: ancient DNA unlocks surprising relationships and phenotypic discordance within the Australo-Pacific Robins

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Abstract Uncertain taxonomy hinders the effective prioritization of taxa for conservation. This problem is acute for understudied island populations in the southwest Pacific Ocean, which are increasingly threatened by habitat loss, predation and climate change. Here, we offer the first test of taxonomic limits and phylogenetic affinities of the iconic Pacific Robin radiation (*Petroica multicolor*) in order to prioritize the conservation of its nominotypical subspecies, the endangered Norfolk Island Robin (*P. m. multicolor*). We integrate phylogenetic analyses of ancient DNA and quantitative measures of plumage and morphometric variation to show that the Norfolk Island Robin should be recognized as a distinct species. Phenotypic and genetic datasets contradict the longstanding treatment of Pacific Robins (including Norfolk Island Robins) and Scarlet Robins (*P. boodang*) as a single species. Instead, we show that Norfolk Island Robins are deeply divergent from Scarlet Robins and have more genetic similarity to Red-capped Robins (*P. goodenovii*) than to other Pacific Robins. This finding is unrepresentative of the current taxonomic and conservation status of the Norfolk Island Robin, which we propose should be recognised as an endemic endangered species. Our study clearly shows that in the absence of contemporary tissues, ancient DNA approaches using historical museum specimens can address taxonomic questions that morphological traits are unable to resolve. Further, it highlights the need for similar studies of other threatened Norfolk fauna with uncertain taxonomic status in order to ensure appropriate conservation prioritization.

Keywords Ancient DNA · Conservation genetics · Endangered species · Insular speciation · Norfolk Island · Pacific

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Introduction

Norfolk Island is a small island located 1500 km off the east coast of Australia in the Pacific Ocean (Fig. 1). Norfolk Island's fauna has suffered high rates of extinction and population decline since European colonization owing to clearance of native habitats for agriculture, habitat degradation by invasive plant and animal species and predation

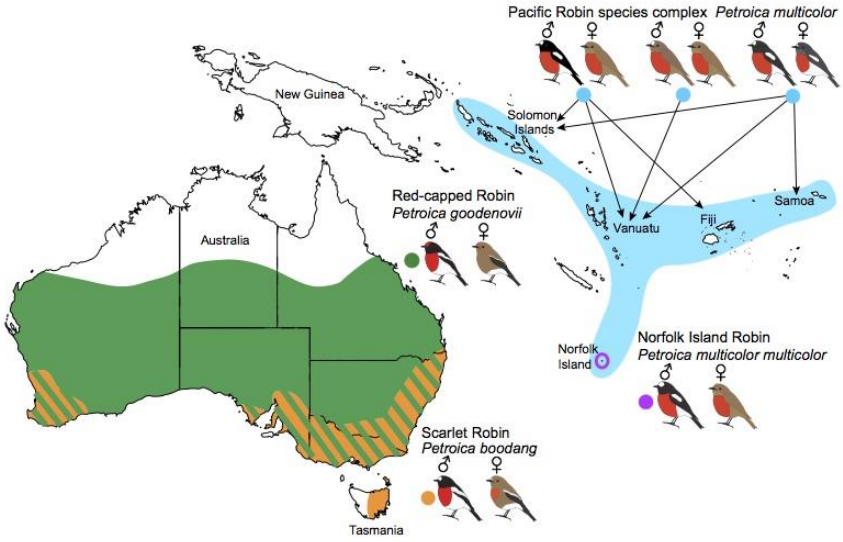


Fig. 1 Geographic range and phenotypic differences of Norfolk Island Robins compared to putative conspecifics and sister taxa. Norfolk Island Robins, Scarlet Robins and Red-capped Robins all exhibit strong sexual dichromatism, while Pacific Robins from the Solomon Islands, Vanuatu, Fiji and Samoa archipelagos exhibit three forms of sexual dichromatism—six subspecies are strongly sexually dichromatic, while the others have reduced sexual dichromatism wherein both sexes either have brown dorsal plumage (three subspecies) or black dorsal plumage (five subspecies). Ranges are redrawn from Schodde and Mason (1999), Boles (2007) and Mayr (1934). Eight other species of *Petroica* are found in Australia, New Zealand and New Guinea (see Boles 2007 for their distributions)

by introduced rats and feral cats (Director of National Parks 2010). Only nine of the fifteen land bird species documented when Europeans settled in 1788 are still extant. Extinctions include the endemic Norfolk Kaka Parrot (*Nestor productus*), Norfolk Island Thrush (*Turdus poliocephalus poliocephalus*), Norfolk Ground Dove (*Gallicolumba norfolciensis*), Long-tailed Triller (*Lalage leucopyga leucopyga*) and Norfolk Boobook Owl (*Ninox novaeseelandiae undulata*) (Schodde and Mason 1999; Director of National Parks 2010; but see Garnett et al. 2011a, b). Recent conservation attempts have halted substantial population decline in the endemic Norfolk Parakeet (*Cyanoramphus cookii*), Norfolk Golden Whistler (*Pachycephala pectoralis xanthoprocta*) and Norfolk Island Robin (*Petroica multicolor multicolor*) (Director of National Parks 2010). However, all remain listed as vulnerable or endangered (Director of National Parks 2010; Garnett et al. 2011b; Department of the Environment 2015).

The taxonomic distinctiveness and phylogenetic relationships of many of Norfolk Island's threatened fauna remains poorly characterized (Schodde et al. 1983; Hermes 1985; Hermes et al. 1986; Schodde and Mason 1999; Christidis et al. 2011; Dutson 2013). This is primarily due to the remoteness of Pacific Ocean islands and the vast geographic scale of Pacific radiations, both of which have hindered the acquisition of tissue samples for molecular analyses thus far. For these reasons, the Norfolk Island subspecies was not included in phylogenetic analyses of the polytypic Pacific Robins (*P. multicolor*; Miller and Lambert 2006; Loynes et al. 2009; Christidis et al. 2011), Golden Whistlers (*P. pectoralis*; Andersen et al. 2014), or Island Thrush (*T. poliocephalus*; Voelker et al. 2007; Jones and Kennedy 2008). Such sampling biases not only perpetuate an incomplete picture of the history of speciation in the Pacific, but they also have ramifications for the conservation of Norfolk Island's threatened fauna.

In this study, we use historical museum samples to achieve complete taxon sampling of the Pacific Robin species complex, *P. multicolor* (Gmelin, 1789), which is an iconic insular radiation that was central to the development of the first theories about island speciation (Mayr 1963; Futuyma 1997; Mayr and Diamond 2001). Our primary goal is to provide a revised taxonomy for Pacific Robins that can be used to prioritize the conservation of its nominotypical subspecies, the endangered Norfolk Island Robin (*P. m. multicolor*).

Norfolk Island Robins are currently declining because of predation from black rats (*Rattus rattus*) and the clearance and degradation of its preferred rainforest habitats on Norfolk Island (Garnett et al. 2011b; Dutson 2013). Population censuses suggest that only 400–500 breeding pairs exist and approximately 80 % of these are restricted to the Norfolk Island National Park that occupies just ~12 % of

the island (Schodde et al. 1983; Hermes 1985; Hermes et al. 1986; Robinson 1988; Major 1989; Robinson 1997; Garnett and Crowley 2000; Commonwealth of Australia 2005; Director of National Parks 2010; Dutson 2013). On this basis Norfolk Island Robins were classified as 'Vulnerable' by the Australian Government EPBC Act 1999 (Department of the Environment 2015) and 'Endangered' under IUCN Red List criteria in the Action Plan for Australian Birds (Garnett et al. 2011b).

Critically, the IUCN considers Norfolk Island Robins as a subspecies of a widespread polytypic species that is of low conservation priority ('Least Concern', BirdLife International 2012). This widespread species (*P. multicolor sens. lat.* following Mayr 1934; Higgins and Peter 2002; Boles 2007) includes fourteen other 'Pacific Robin' subspecies as well as three subspecies of 'Scarlet Robin' from mainland Australia. However, this taxonomy is contentious owing to (1) morphological differences between Australian and Pacific forms that are argued to distinguish Scarlet Robins (*P. boodang*) and Pacific Robins (*P. multicolor*) as separate species (Schodde and Mason 1999), and (2) recent mtDNA evidence from a single Pacific Robin from the Solomon Islands that suggests Pacific Robins could be sister to the phenotypically disparate Red-capped Robins (*P. goodenovii*) of mainland Australia rather than Scarlet Robins as long hypothesised (Christidis et al. 2011) (Fig. 1). There is also ambiguity about whether morphometrics and plumage traits align Norfolk Island Robins with Scarlet Robins or with the other Pacific Robin subspecies from the Solomon Islands (including Bougainville island), Vanuatu, Fiji and Samoa (henceforth referred to as 'Pacific Island Robins') (Schodde and Mason 1999).

To resolve these outstanding taxonomic issues, we use ancient DNA from museum skins and quantitative analysis of plumage colour and morphometrics to assess the overall distinctiveness of the Norfolk Island Robin, and to evaluate three hypotheses from the literature:

(1) Norfolk Island Robins are most closely related to Pacific Island Robins to the exclusion of all other taxa (i.e., they are reciprocally monophyletic) (*P. multicolor* sensu Schodde and Mason 1999).
(2) Scarlet Robins, Pacific Island Robins and Norfolk Island Robins are most closely related to each other (*P. multicolor* sensu Mayr 1934; Boles 2007; BirdLife International 2012).
(3) Red-capped Robins, Pacific Island Robins and Norfolk Island Robins are most closely related to each other (Christidis et al. 2011).

We present these as three alternatives as these are three hypotheses that are in the published literature. However, they are not necessarily mutually exclusive. For example, if H1 is true, then the next closest taxon could be either the Scarlet Robin (H2) or Red-capped Robin (H3). Conversely, H2 or H3 could be true even if H1 is not.

By producing the first phylogeny with complete taxonomic sampling of this iconic Pacific radiation we aim to contribute to a growing body of data on the historical biogeography of Pacific Ocean birds (Clegg et al. 2002; Estoup and Clegg 2003; Filardi and Moyle 2005; Moyle et al. 2009; Uy et al. 2009a, b; Andersen et al. 2013; Irestedt et al. 2013; Andersen et al. 2014). In particular, we offer one of the few molecular tests of the historical relationships among Norfolk Island forms and their Australian mainland and Pacific Ocean congeners (for other examples see Norman et al. 1998; Boon et al. 2001; Estoup and Clegg 2003; Goldberg et al. 2011).

Methods

Genetic distinctiveness

Specimens and loci sampled

No contemporary tissues were available for the Norfolk Island Robin (NI) owing to its remote location and endangered conservation status. For similar reasons, contemporary tissue samples were only obtained for 14 Pacific Island Robins (PI) from the Solomon Islands (2/4 subspecies sampled), Vanuatu (1/6 subspecies sampled) and Fiji (2/3 subspecies sampled). No contemporary tissues were available from the archipelago of Samoa (0/1 subspecies sampled). Thus, we used small subsamples from museum study skins collected between 1912 and 1953 to achieve complete taxon sampling of NI Robins and PI Robins. Most of these were collected as part of the Whitney South Seas Expedition of the American Natural History Museum and were used in the taxonomic revision of this species complex by Mayr (1934) (LeCroy 2008). We sampled a total of 5 NI Robins and 36 PI Robins (14 contemporary tissues, 22 historical museum samples). The specific number we sampled per archipelago and subspecies was (epithets only used for brevity): 5 from Norfolk Island: *multicolor* n = 5; 10 from Solomon Islands: *polymorpha* n = 3, *kulambangrae* n = 3, *dennisi* n = 2, *septentrionalis* n = 2; 17 from Vanuatu: *soror* n = 3, *ambrynensis* n = 3, *feminina* n = 3, *cognata* n = 3, *similis* n = 3, *tannensis* n = 2; 7 from Fiji: *becki* n = 2, *kleinschmidti* n = 3, *taveunensis* n = 2; 2 from Samoa: *pusilla* n = 2).

We use a mitochondrial *ND2* dataset derived from historical museum samples and contemporary tissues of NI Robins and PI Robins sequenced for this study. *ND2* sequences from six other species of *Petroica* were obtained from GenBank (see Online Appendix 1 for sample details).

Historical museum samples were only sequenced for *ND2* owing to the poorer quality of DNA. However, we used the fresh tissue samples to further explore support for the phylogenetic relationships indicated by *ND2* using mitochondrial *CO1*, autosomal nuclear *CLOCK* (intron 10) and Z-linked nuclear *ACO1* (intron 9) (see Online Appendix 1 for sample details). All sequences were deposited in GenBank (accession numbers KT372722-KT372779 and KT388359-KT388384).

DNA extraction and sequencing

ND2 was sequenced from 22 historical museum samples (toepads) at the Australian Centre for Ancient DNA, University of Adelaide and from 14 contemporary tissue samples at the University of Maryland, Baltimore County using the DNA extraction and sequencing protocols described in Kearns et al. (2015). *CO1* and the two nuclear introns were sequenced from contemporary tissue samples using a high-throughput 96-well plate format. Muscle and liver samples were digested overnight at 55 °C in a standard proteinase-k buffer and extracted with a phenol–chloroform protocol as implemented on an Autogen Prep 965 extraction robot (AutoGen, Massachussetts). An additional two samples of New Zealand Tomtit *Petroica macrocephala* (UCA104713, UCA104714) were extracted separately using a DNeasy Tissue Kit (Qiagen, Valencia, California, USA).

The DNA barcode region (652 bp) of the 5′ end of the cytochrome oxidase subunit 1 gene (COI) was amplified using primers BirdF1 and BirdR1 (Hebert et al. 2004). Each 10 μL reaction mix contained 0.3 μL of each 10 μM primer, 0.5 μL of dNTP mix (containing 2.5 mM of each nucleotide), 0.1 μL Biolase Taq polymerase (Bioline), and 2.0 mM $MgCl_2$. Thermocycler conditions were an initial denaturation step of 94 °C for 4 min followed by thirty-five cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 45 s and a final 3 min period at 72 °C. PCR amplification of nuclear *ACO1intron 9* and *CLOCK intron 10* used primers ACO1.9F, ACO1.9R, CLK.10F and CLK.10R (Kimball et al. 2009) and was performed in 15ul reactions using 2 μl 10 × buffer, 1.25 μl MgCl, 1.2 μl dNTP (10 μM), 0.4 μl each primer (10 μM), 0.15 μl Biolase Taq (Bioline), 1.2 μl DNA template and 8.4 μl H_20. Thermocycling conditions for the introns were the same as for COI except an annealing temperature of 55 °C was used, the extension stage of each cycle was 1 min long, and forty-five cycles were run for *CLOCK*. All PCR products were cleaned using the ExoSapIT (Affymetrix) protocol, and sequenced in both directions using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) with 30 cycles of sequencing and an annealing temperature of 50 °C. Sequences were edited and aligned using Sequencher 4.7 (Gene Codes, Ann Arbor, MI), and the absence of indels and stop codons was confirmed for *CO1* sequences. Polymorphic SNPs in *ACO1* and *CLOCK* were coded with IUPAC ambiguity codes for phylogenetic analyses. Multinucleotide indels were considered to result from a single insertion-deletion event and were reduced to 1 bp. Two indels in ACO1 were completely deleted owing to alignment issues.

Phylogenetic analysis

ND2 sequences derived from contemporary tissues, historical samples and GenBank were assembled and aligned using the MUSCLE algorithm (Edgar 2004) in MEGA v6 using default settings (Tamura et al. 2011). The final *ND2* alignment contained 940 bp and 70 individuals across seven named species of *Petroica*. We rooted the *ND2* phylogeny using sequences from *Pachycephalopsis, Amalocichla* and *Eugerygone* given that phylogenetic analyses of *CO1, ND2* and *beta-fibrinogen intron 5* place them as sisters to *Petroica* (Christidis et al. 2011). *ACO1* and *CLOCK* were sequenced for twenty-eight individuals from seven species of *Petroica*. Nuclear introns were concatenated and the resulting alignment was 1256 bp in length. MtDNA *CO1* was sequenced for the same 28 individuals and the final alignment was 575 bp. Both datasets used *Eugerygone* as an outgroup (Christidis et al. 2011).

Phylogenetic relationships were estimated using Bayesian inference implemented in MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001). Preliminary MrBayes analyses using 50 million generations and a 25 % burnin showed that the default heating temperature of T = 0.2 resulted in poor swap rates between heated and cold chains (0–14 %). Thus, we ran our final Bayesian analyses using a heating temperature of T = 0.1, which offered better swap rates between the four chains. We ran each analysis four times for 5×10^7 generations, sampling every 100 generations and using the best partitioning scheme and substitution model selected by PartitionFinder v1.1.1 (Lanfear et al. 2012) under the BIC—*ND2* dataset partitioned by codon: 1st codon GTR+I+G, 2nd codon HKY+I+G, 3rd codon GTR+G; *CO1* dataset partitioned by codon: 1st codon K80+I+G, 2nd codon HKY+I, 3rd codon GTR+G; concatenated nuclear dataset: *CLOCK*, HKY; *ACO1*, HKY. We assessed mixing and convergence of parameters across the four independent Bayesian runs by ensuring that posterior density plots showed no obvious trends and that the independent runs did not differ significantly under a Bayes factor threshold of $2\log_{10}BF = 6$ (Kass and Raftery 1995). Using these criteria we excluded the first 4×10^7 generations as burnin, which resulted in a post-burnin sample of

10 million generations (100,000 trees) from which we calculated 50 % majority rule consensus trees.

We compared Bayesian phylogenies for the *ND2, CO1* and nuclear datasets to those estimated using maximum likelihood implemented in RAxML 7.0.3 (Stamatakis 2006) under the 'fast ML' algorithm with 1000 bootstrap pseudoreplicates and with the most complex substitution model offered, GTRGAMMA, imposed for all loci and partitions. We also calculated the average number of nucleotide substitutions per site (*Dxy*) and the number of fixed differences between populations for *ND2* using DnaSP v5.10 (Rozas et al. 2003). One Pacific Robin sequence from Samoa (AMNH 129055) was omitted from the DnaSP analyses owing to missing data.

Phylogenetic hypothesis testing

We used Bayes factors (Kass and Raftery 1995; Nylander et al. 2004) to discriminate between the phylogenetic hypothesis obtained from Bayesian analyses of *ND2* without topological constraints and alternative phylogenetic hypotheses obtained using topological constraints under the identical settings in MrBayes. We evaluated the following hypotheses: (1) NI Robins and PI Robins are most closely related to each other to the exclusion of all other taxa (i.e., they are reciprocally monophyletic) (*P. multicolor* sensu Schodde and Mason 1999), (2) Scarlet, PI and NI Robins are reciprocally monophyletic (*P. multicolor* sensu Mayr 1934; Boles 2007; BirdLife International 2012), and (3) Red-capped, PI and NI Robins are reciprocally monophyletic (observed unconstrained phylogeny, see Results) (Christidis et al. 2011). We also evaluated support for topologies where the monophyly of all NI specimens was constrained, and where NI was sister to either Solomon Islands or Vanuatu/Fiji/Samoa or Red-capped Robin lineages owing to rooting issues encountered in the unconstrained *ND2* phylogeny (see Results). Bayes factors were calculated from the post-burnin harmonic means of the marginal likelihoods from the constrained and unconstrained *ND2* phylogenies using a threshold value of $2\log_{10}BF = 10$ as decisive evidence against the alternative hypotheses represented by the constrained analyses (Kass and Raftery 1995; Nylander et al. 2004).

Phenotypic distinctiveness

Morphometrics

We measured wing length, tail length and bill length for 46 NI Robins (27 males, 19 females), 186 PI Robins (116 males, 70 females), 33 Red-capped Robins (17 males, 16 females) and 34 Scarlet Robins (19 males, 15 females) following the methods described in Kearns et al. (2015) (see Online Appendix 2 for sample details). This included all described subspecies of PI Robins and Scarlet Robins and geographic representatives of monotypic Red-capped Robins (Schodde and Mason 1999). Principal components analysis (PCA) was performed for each sex in order to condense morphometric measurements and get an estimate of overall "body size". In both sexes, PC1 explained the majority of the variation (62.3 % male, 63.3 % female; eigenvalue: 1.87 male, 1.90 female) and had highest loadings for wing and tail lengths, while PC2 explained a smaller proportion of the variation (31.9 % male, 28.2 % female; eigenvalue: 0.96 male, 0.85 female) and had high loadings for bill length. We then explored variation in wing, tail and bill length, and the first two principal components using ANOVAs and Tukey–Kramer honestly significant difference (HSD) for post hoc pairwise comparisons of means. We applied a Bonferroni correction on each trait to adjust the significance level (α) for multiple comparisons between the four taxa and two sexes (α 0.05/6 = 0.008). All statistics were performed in JMP 10 (SAS Inc., Cary, NC).

Plumage colour

The spectral reflectance of adult male breast plumage of Scarlet (n = 10), Red-capped (n = 10), Norfolk Island (n = 24) and Pacific Island Robins (n = 102) was measured using an Ocean Optics USB2000 reflectance spectrophotometer following Kearns et al. (2015) (see Online Appendix 2 for sample details). Two statistical approaches were used to test for spectral differences. First, we performed a principal components analysis on the reflectance curves in order to condense the 77 reflectance measures between 320 and 700 nm into two principal components (PCs). PC1 explained 59.9 % of the variation, had an eigenvalue of 46.1 and had highest loadings for reflectance measures between 320 and 600 nm (uv/violet and yellow). PC2 explained 32.4 % of the variation, had an eigenvalue of 24.9 and had highest loadings for reflectance measures between 580 and 700 nm (yellow and red). Second, we calculated brightness "B1", saturation/chroma "S5a" and hue "H4a" (Hill and McGraw 2006) for each individual using the program CLR v1.05 (Montgomerie 2008). Spectral differences between Scarlet, Red-capped, NI and PI Robins were explored using ANOVAs and Tukey–Kramer HSDs for post hoc pairwise comparisons of means. We applied a Bonferroni correction on each colour variable to adjust the significance level (α) for multiple comparisons between the four taxa (α 0.05/4 = 0.013). Mean reflectance curves were also calculated for Scarlet, Red-capped, Norfolk Island, and Pacific Island Robins. All statistics were performed in JMP 10 (SAS Inc., Cary, NC).

Results

Phylogenetic analysis

No premature stop codons, indels or polymorphic base pairs were observed in the *ND2* and *CO1* mtDNA datasets. *ND2* sequences from historical samples showed no evidence of contamination, and they grouped with sequences amplified from contemporary tissues from the same archipelago and/or subspecies.

Norfolk Island (NI) Robins were genetically distinct from all other *Petroica* robins (Fig. 2). We found no shared *ND2* haplotypes among NI Robins and other species of *Petroica* and subspecies of Pacific Island (PI) Robins. NI Robins were 1.8 % divergent from the Red-capped Robin, and 3.3 % divergent from PI Robins. In contrast, Norfolk Island Robins were 11.3 % divergent from Scarlet Robins with whom they have long been treated as conspecific. Although both ML and Bayesian inference failed to place the five *ND2* sequences from the NI Robin in a monophyletic clade (Fig. 2), this seems to be an issue with the mis-placement of the root of the NI+PI+Red-capped clade within NI Robins. The NI Robin *ND2* sequences differ from each other by just one base pair, and NI Robins form a distinct group in the unrooted phylogeny (Fig. 2b). Bayes factors support this explanation, as the *ND2* phylogeny enforcing the monophyly of NI Robins had a nearly identical score to the unconstrained phylogeny (harmonic mean unconstrained = −5240.93, harmonic mean NI monophyly constrained = −5240.92, $2\log_{10}BF = 0.02$).

Phylogenetic analyses of two mtDNA loci (*ND2, CO1*) and two nuclear loci (*ACO1, CLOCK*) supported the close relationship of Pacific Robins and Red-capped Robins to the exclusion of Scarlet Robins (Fig. 3; 99–100 % ML bootstrap, 1.0 Bayesian posterior probability). Three primary lineages were present in each dataset: (1) New Zealand: Tomtit *P. macrocephala*, (2) Australian: Pink Robins *P. rodinogaster*, Rose Robins *P. rosea*, Flame Robins *P. phoenicea* and Scarlet Robins, and (3) Pacific: NI, PI and Red-capped Robins (Figs. 2, 3). Relationships among these three main lineages were poorly resolved for mtDNA, but the concatenated nuclear dataset supported a sister relationship between the New Zealand Tomtit and the four species in the 'Australian' lineage (Figs. 2, 3). The *ND2* dataset strongly supported the monophyly of PI Robins, as well as the division of this lineage into two well supported clades—one containing the four subspecies from the Solomon Islands, and the other containing the ten subspecies from the archipelagos of Vanuatu, Fiji and Samoa (Fig. 2). *CO1* and nuclear phylogenies were less-well resolved/supported but consistent with the genetic differentiation of the Solomon Islands and Vanuatu/Fiji/Samoa lineages, and the monophyly of PI Robins (Fig. 3).

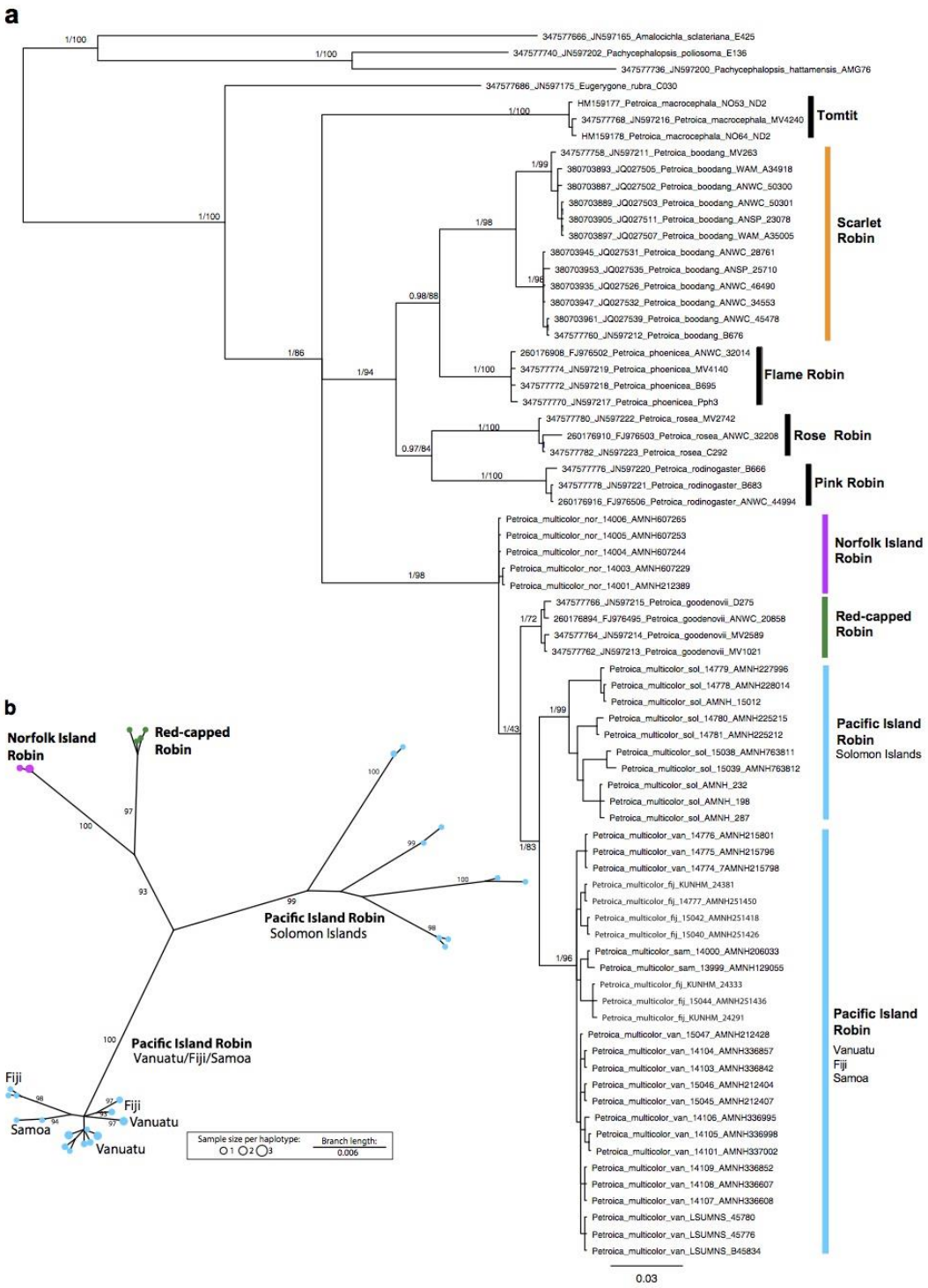
Fig. 2 Genetic distinctiveness and phylogenetic affinities of the Norfolk Island Robin. **a** ND2 phylogeny of Australo-Pacific Robins showing support for the sister relationship of Norfolk Island Robins, Pacific Island Robins and Red-capped Robins to the exclusion of Scarlet Robins. The Bayesian consensus tree is shown with Bayesian posterior probabilities and maximum likelihood bootstrap support indicated for each node. **b** Unrooted maximum likelihood tree of the 'Pacific' clade showing the close phylogenetic relationship of Norfolk Island Robins and Red-capped Robins to the exclusion of the Solomon Island and Vanuatu/Fiji/Samoa lineages of Pacific Island Robins

Our hypothesis tests using Bayes factors offered strong support for our finding that NI Robins form a clade with Red-capped and PI Robins (Hypothesis 3: Fig. 2; harmonic mean unconstrained = −5240.93). The unconstrained *ND2* phylogeny was significantly different to the other alternative hypotheses from the literature wherein (1) NI Robins and PI Robins are each other's closest relatives (Hypothesis 1: *P. multicolor* sensu Schodde and Mason 1999; harmonic mean NI+PI constrained = −5249.43, $2\log_{10}BF = 17$) and (2) NI Robins, PI Robins and Scarlet Robins are each other's closest relatives (Hypothesis 2: *P. multicolor* sensu Mayr 1934; harmonic mean NI+PI+Scarlet constrained = −5373.60, $2\log_{10}BF = 265.34$). Bayes factor tests found that enforcing a sister relationship between NI Robins and Red-capped Robins resulted in a significantly different phylogeny from the unconstrained phylogeny (harmonic mean NI+Red-capped constrained = −5247.05, $2\log_{10}BF = 12.24$). This is despite genetic divergence (Dxy 1.8 %) and clustering in the un-rooted phylogeny (Fig. 2b) both suggesting that NI Robins are more closely related to Red-capped Robins than to PI Robins. Phylogenies enforcing a sister relationship between NI Robins and either the Solomon Island or Vanuatu/Fiji/Samoa lineages were also significantly different from the unconstrained phylogeny (harmonic mean NI+Solomons constrained = −5252.03, $2\log_{10}BF = 22.2$; harmonic mean NI+Vanuatu/Fiji/Samoa constrained = 5250.24, $2\log_{10}BF = 18.62$).

Phenotypic distinctiveness

Morphometrics

Scarlet, Red-capped and PI Robins each showed sexual dimorphism in wing length (Scarlet: $t_{(32)} = 3.33$, $p = 0.002$; Red-capped: $t_{(31)} = 5.46$, $p < 0.0001$; PI: $t_{(184)} = 3.91$, $p < 0.0001$) (Fig. 4). NI Robins did not show sexual dimorphism in wing length after Bonferroni correction ($\alpha = 0.008$) (NI: $t_{(44)} = 2.51$, $p = 0.016$). Only Scarlet Robins were sexually dimorphic for tail length after Bonferroni correction (Scarlet: $t_{(32)} = 3.25$, $p = 0.003$; Red-capped: $t_{(31)} = 2.21$, $p = 0.035$; NI: $t_{(44)} = 1.50$, $p = 0.14$; PI: $t_{(183)} = 0.73$, $p = 0.47$). No differences between the sexes were observed


a
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347577686_JN597175_Eugerygone_rubra_C030
HM159177_Petroica_macrocephala_NO53_ND2
347577768_JN597216_Petroica_macrocephala_MV4240
HM159178_Petroica_macrocephala_NO64_ND2
Tomtit
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Scarlet Robin
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347577770_JN597217_Petroica_phoenicea_Pph3
Flame Robin
347577780_JN597222_Petroica_rosea_MV2742
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347577782_JN597223_Petroica_rosea_C292
Rose Robin
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347577778_JN597221_Petroica_rodinogaster_B683
260176916_FJ976506_Petroica_rodinogaster_ANWC_44994
Pink Robin
Petroica_multicolor_nor_14006_AMNH607265
Petroica_multicolor_nor_14005_AMNH607253
Petroica_multicolor_nor_14004_AMNH607244
Petroica_multicolor_nor_14003_AMNH607229
Petroica_multicolor_nor_14001_AMNH212389
Norfolk Island Robin
347577766_JN597215_Petroica_goodenovii_D275
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347577764_JN597214_Petroica_goodenovii_MV2589
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Red-capped Robin
Petroica_multicolor_sol_14779_AMNH227996
Petroica_multicolor_sol_14778_AMNH228014
Petroica_multicolor_sol_AMNH_15012
Petroica_multicolor_sol_14780_AMNH225215
Petroica_multicolor_sol_14781_AMNH225212
Petroica_multicolor_sol_15038_AMNH763811
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Petroica_multicolor_sol_AMNH_232
Petroica_multicolor_sol_AMNH_198
Petroica_multicolor_sol_AMNH_287
Pacific Island Robin
Solomon Islands
Petroica_multicolor_van_14776_AMNH215801
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Petroica_multicolor_van_14774_7AMNH215798
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Petroica_multicolor_van_LSUMNS_45776
Petroica_multicolor_van_LSUMNS_B45834
Pacific Island Robin
Vanuatu
Fiji
Samoa
0.03
b
Norfolk Island Robin
Red-capped Robin
Pacific Island Robin
Solomon Islands
Pacific Island Robin
Vanuatu/Fiji/Samoa
Fiji
Samoa
Fiji
Vanuatu
Vanuatu
Sample size per haplotype: 1 2 3
Branch length: 0.006

in bill length (Scarlet: $t_{(31)} = 0.080$, $p = 0.94$; Red-capped: $t_{(29)} = 1.64$, $p = 0.11$; NI: $t_{(43)} = 0.23$, $p = 0.82$; PI: $t_{(169)} = 2.19$, $p = 0.030$) (Fig. 4).

All morphometric variables, as well as PC1 ("overall body size") and PC2 ("bill length"), were significantly differentiated across Scarlet, Red-capped, NI and PI Robins (ANOVA all $p < 0.0001$; see Fig. 4 for ANOVA F statistics for each variable and each sex). *Post-hoc* pairwise comparisons showed that NI Robins had significantly longer wings, tails and bills compared to PI Robins (Tukey's HSD: $p < 0.0001$ in all pairwise comparisons for both sexes). The only non-significant comparison between NI Robins and Scarlet Robins was for tail length in both males ($p = 0.8$) and females ($p = 0.98$). Only wing length in females was non-significant between PI Robins and Red-capped Robins ($p = 0.99$). The larger body size of NI Robins approached that of Scarlet Robins, whereas the smaller size of PI Robins approached Red-capped Robins (Fig. 4). Despite differences in size, however, NI and PI Robins had significantly larger bills compared to the mainland species they were closest to in body size (Tukey's HSD: $p < 0.0001$ for PI vs. Red-capped and NI vs. Scarlet). As a result of these different patterns of body size and bill length, NI Robins occupied distinct morphometric PCA space compared to PI, Scarlet and Red-capped Robins (Fig. 4f). In contrast, PI Robins and Red-capped Robins overlapped in both body size and bill length (Fig. 4d–f).

Plumage colour

Estimates of the brightness, saturation and hue (and PC1 and PC2) of male breast plumage were significantly differentiated across Scarlet, Red-capped, NI and PI Robins (ANOVA $p < 0.0001$ for all comparisons; see Fig. 5 for individual ANOVA F statistics). *Post-hoc* pairwise comparisons of NI and PI Robins using Tukey's HSD showed significant differences for saturation ($p < 0.0001$), hue ($p < 0.0001$), brightness ($p = 0.011$) and PC2 ($p < 0.0001$), but not PC1 ($p = 0.68$) after Bonferroni correction ($\alpha = 0.013$). In contrast, only one variable each was significantly different between NI Robins and Scarlet Robins (Tukey's HSD: Hue: $p = 0.008$), and NI Robins and Red-capped Robins (Tukey's HSD: PC1: $p = 0.012$). PI Robins were on average darker and more saturated than the three other taxa (Fig. 5a, b). This trend was also apparent in the mean reflectance curve for PI Robins, which showed less reflectance between 600 and 700 nm within the orange-red reflectance range (Fig. 5e), as well as the significant differentiation of PI Robins from all other taxa at PC2 (Tukey's HSD: all $p < 0.0001$), which had highest loadings at these higher wavelengths (Fig. 5e). PI Robins also showed more

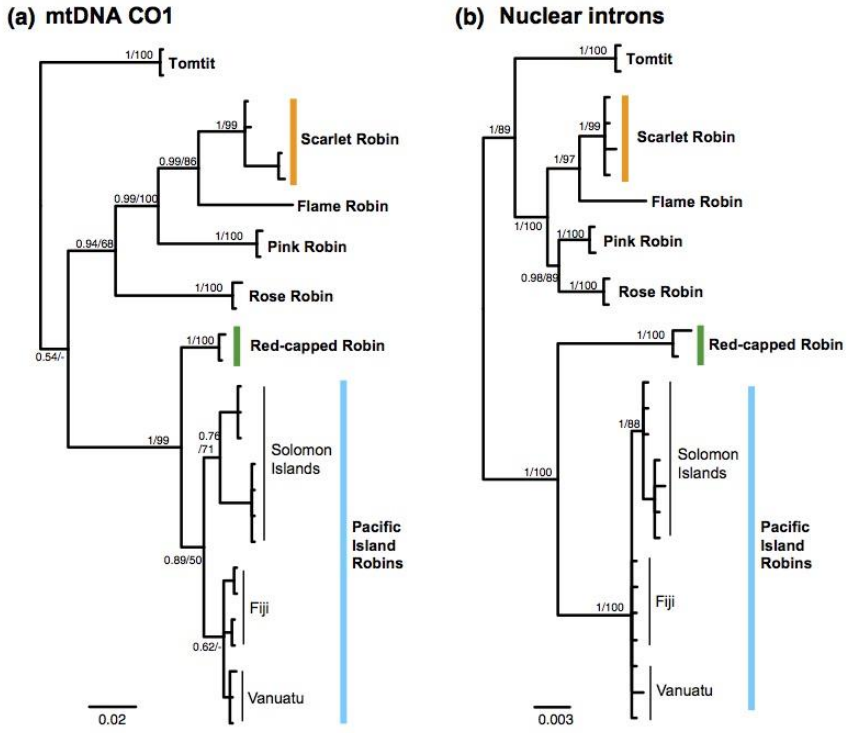

Fig. 3 Phylogenetic analysis of CO1 and nuclear introns of Australo-Pacific Robins showing multilocus support for the sister relationship of Pacific Island Robins and Red-capped Robins to the exclusion of Scarlet Robins. Note that Norfolk Island Robins were not sequenced for these loci as no contemporary tissue samples were available for this taxon. Bayesian consensus trees are shown for both datasets with Bayesian posterior probabilities and maximum likelihood bootstrap support indicated for each node. *Eugerygone rubra* (MV C030) was used to root the trees

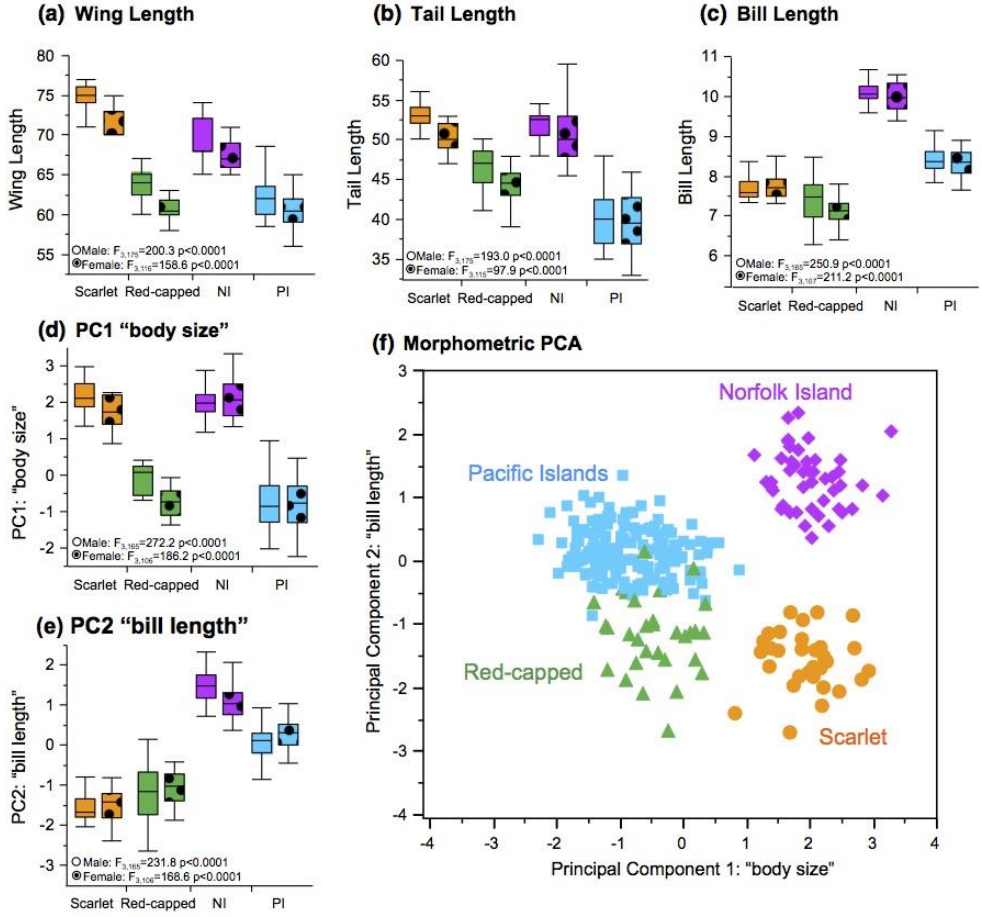

Fig. 4 Analysis of morphometric variation illustrating the distinctiveness of Norfolk Island Robins. **a–e** Variation in wing, tail and bill lengths, as well as the first two principal components for males (solid) and females (pattern) of all four taxa. *Box plots* represent the minimum, 25 % quantile, median, 75 % quantile and maximum for each variable. **f** Principal components analysis (PCA) showing the differentiation of Scarlet Robins (*circles*), Red-capped Robins (*triangles*), Norfolk Island Robins (*diamonds*) and Pacific Island Robins (*squares*) across the first two principal components, which had highest loadings for bill length (PC2) and wing and tail length (*body size*) (PC1). Males and females did not show major differences in morphometric measurements (see **a–e**) thus for simplicity they are presented together in (**f**). See Online Appendix 1 for details of sample sizes and collecting localities

variability in breast colour than the three other taxa (Fig. 5d). This is unsurprising given that fourteen diversely plumaged subspecies are represented in this group (Mayr 1934; Kearns et al. 2015). However, examining patterns of variation across the range of PI Robins was beyond the scope of this paper.

Discussion

Our data overwhelmingly reject the taxonomic treatment of the Norfolk Island (NI) Robin as a weakly differentiated subspecies of either the Scarlet Robin (Hypothesis 2: *P. multicolor* includes NI+PI+Scarlet sensu Mayr 1934; Higgins and Peter 2002; Boles 2007; BirdLife International 2012) or the Pacific Robin (Hypothesis 1: *P. multicolor* includes NI+PI sensu Schodde and Mason 1999). Instead, our phylogenetic and quantitative phenotypic analyses using complete taxon sampling of the Pacific robin radiation strongly support the distinctiveness of the Norfolk Island Robin at the species-level, as well as its phylogenetic placement in a 'Pacific' clade containing Pacific Island Robins and Red-capped Robins (Hypothesis 3: Fig. 2; Christidis et al. 2011). Recognizing the Norfolk Island Robin as a distinct species requires a revision of the taxonomy of the Pacific robin radiation, as well as a re-

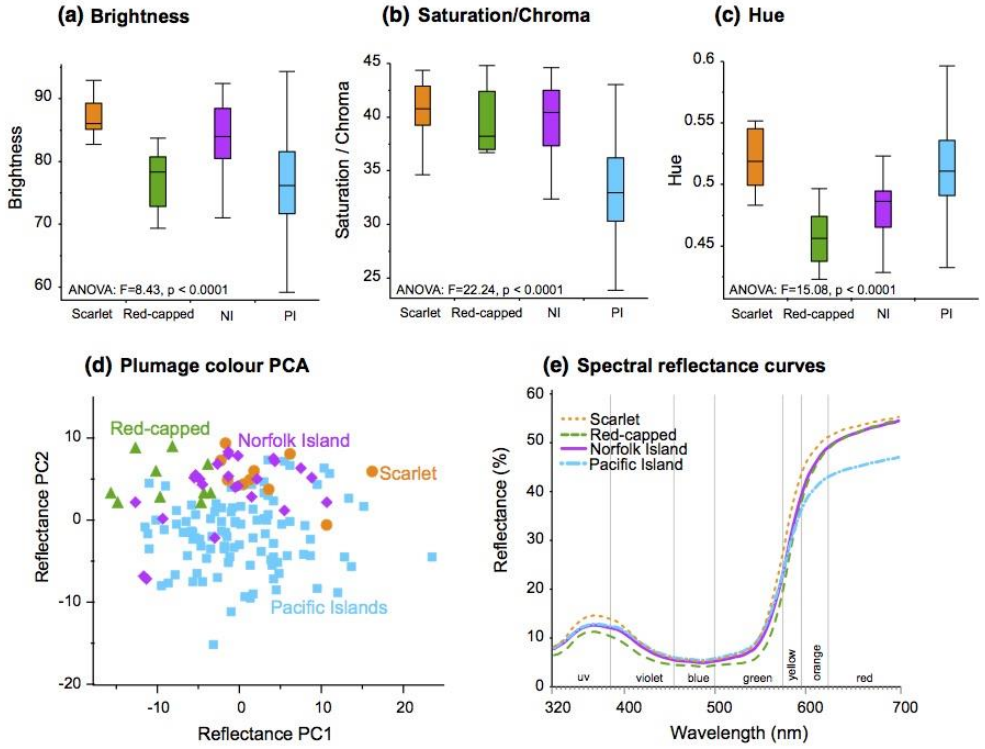

Fig. 5 Analysis of variation in breast plumage among males of Norfolk Island Robins, Pacific Island Robins, Red-capped Robins and Scarlet Robins across three measures of *colour* (**a** brightness, **b** saturation, **c** hue), two principal components (**d**) and mean spectral reflectance curves for each taxon (**e**). Both principal components showed significant variation among the four taxa (PC1 ANOVA: $F = 7.57$, $p < 0.0001$, PC2 ANOVA: $F = 23.39$, $p < 0.0001$). *Box plots* in (**a**–**c**) depict the minimum, 25 % quantile, median, 75 % quantile and maximum for each variable. Points in (**d**) are calculated from the mean percent reflectance measures for each individual, and symbols follow Fig. 4. See Online Appendix 1 for details of sample size and collection locality. (Color figure online)

assessment of the conservation status and management plan of the endangered Norfolk Island Robin (Director of National Parks 2010; Garnett et al. 2011b; Dutson 2013).

Paraphyly of the iconic Pacific robin radiation

We find clear evidence for at least three distinct lineages within the 'Pacific' clade (Red-capped Robins, Norfolk Island Robins, Pacific Island Robins; Figs. 2, 3). However, the sequence of divergence among these lineages is unclear. Either Norfolk Island Robins and Red-capped Robins are sisters—as indicated by the unrooted *ND2* phylogeny (Fig. 2b) and contrasting measures of genetic divergence between NI and Red-capped Robins ($Dxy = 1.8$ %) and NI and PI Robins ($Dxy = 3.3$ %)—or Norfolk Island Robins are sister to a clade containing the smaller-bodied Red-capped Robins and Pacific Island Robins—as indicated by ML and Bayesian phylogenetic analyses (Fig. 2a) and post hoc hypothesis testing using Bayes factors. There is also some indication that robins from the Solomon Islands and the archipelagos of Vanuatu, Fiji and Samoa form two distinct lineages ($Dxy = 3.8$ %) (Figs. 2, 3) that might not be each other's closest relatives (Fig. 2b). Examining patterns of variation across the range of Pacific Island Robins was beyond the scope of this paper and will be explored elsewhere. Important here is that we find no support for a close sister relationship between Norfolk Island Robins and Pacific Island Robins (Fig. 2a, b) and strong support for the paraphyly of Pacific Robins as currently circumscribed (i.e., either NI+PI sensu Schodde and Mason (1999) and Christidis and Boles (2008) or NI+PI+Scarlet sensu Mayr (1934) and Boles (2007)) owing to the presence of the Red-capped Robin nested within the 'Pacific' clade (Fig. 2a, b).

Contemporary tissues were not available for the endangered Norfolk Island Robin population, thus our findings rely on mitochondrial *ND2* sequenced using ancient DNA techniques from historical museum

specimens collected by the American Museum of Natural History between 1912 and 1926. We also used ancient DNA to obtain complete sampling of all recognized subspecies of Pacific robins. Inferences based on a single, maternally inherited marker can sometimes be misled by gene flow and incomplete lineage sorting. However, several lines of evidence suggest that our dataset does not have these issues. First, there are substantial oversea distances between Norfolk Island Robins and the rest of *Petroica*, suggesting that gene flow is implausible albeit not impossible (Fig. 1). Second, our finding of distinct Norfolk, Solomons and Vanuatu/Fiji/Samoa lineages is concordant with biogeographic expectations and observations in other Pacific biota. Third, consistent phylogenetic hypotheses for *Petroica* have been recovered across several different studies employing different taxon and locus sampling (Miller and Lambert 2006; Loynes et al. 2009; Christidis et al. 2011; this study). Fourth, phylogenetic analyses of mtDNA *CO1* and two nuclear introns for taxa with contemporary tissues are congruent with the *ND2* phylogeny (Figs. 2, 3). Fifth, *ND2* from the five subspecies of Pacific Island Robin sequenced from both ancient and contemporary tissues grouped together. Finally, the distinctiveness of the Norfolk Island Robin at *ND2* is accompanied by marked phenotypic distinctiveness (Figs. 4, 5). Together these lines of evidence give confidence in the *ND2* results, and strongly support the recognition of the Norfolk Island Robin as a distinct species.

Taxonomic revision

The lack of reciprocal monophyly of Pacific Robins (i.e., NI+PI sensu Schodde and Mason 1999; Christidis and Boles 2008) dictates that the Norfolk Island Robin (*P. m. multicolor*), Pacific Island Robin (*P. multicolor subsps.*) and Red-capped Robin (*P. goodenovii*) must either be treated as a single species, or, as appears to have more merit, as multiple separate species. If these forms are to be treated as a single species, *Petroica multicolor* (Gmelin, 1789) has priority over *Petroica goodenovii* (Vigors & Horsfield, 1827). However, we argue that these lineages have sufficiently distinct morphology and *ND2* sequences to warrant species-status under multiple species criteria. Specifically:

1. The level of divergence in *ND2* of Norfolk Island and Pacific Island Robins (3.3 %) implies that these lineages have a long independent evolutionary history. Norfolk Island and Pacific Island Robins were also substantially differentiated in bill and body size (Fig. 4), as well as in brightness, saturation and hue of male breast plumage (Fig. 5). The level of phenotypic distinctiveness between the two is comparable to that observed between other genetically divergent *Petroica* species that have similar plumage (e.g., Scarlet Robin versus either PI or NI Robins, Fig. 4; Rose Robins *P. rosea* versus Pink Robins *P. rodinogaster*, Schodde and Mason 1999), and their morphometric divergence is greater than that found between PI Robins and Red-capped Robins (Fig. 4). Together these data provide a strong argument for naming Norfolk Island Robins and Pacific Island Robins as separate species.
2. Norfolk Island and Red-capped Robins were 1.8 % divergent at *ND2* and were substantially distinct in phenotypic traits such as body size, bill length (Fig. 4), the colour of the forehead spot (Fig. 1) and life history traits (Higgins and Peter 2002). No author has lumped these two species taxonomically or suggested in the literature that they might be sister taxa prior to molecular analysis (Schodde and Mason 1999; Higgins and Peter 2002; Boles 2007; BirdLife International 2012; Christidis and Boles 2008; Christidis et al. 2011; Garnett et al. 2011b). We see little benefit in combining these two species as a single species, and argue instead that all lines of evidence are consistent with the species level distinctiveness of the Norfolk Island Robin.

Treatment of Norfolk Island, Pacific Island and Red-capped Robins as separate species is consistent with modern standards in bird taxonomy and with the diagnostic criteria of the Evolutionary, Phylogenetic and General Lineage Species Concepts (reviewed in Wheeler and Meier 2000; de Queiroz 2005; Gill 2014; see also Tobias et al. 2010), as well as the Biological Species Concept if the updated alternative null hypothesis of Gill (2014) is applied. *Petroica multicolor* (Gmelin, 1789) has priority for the populations on Norfolk Island, and *Petroica pusilla* Peale, 1848 has priority for the rest of the populations in the southwest Pacific. We recommend the English names of Norfolk Island Robin and Pacific Island Robin, respectively, for these two species, and the use of 'Pacific robin' when referring to the entire species complex including Red-capped Robins. Our data also show that *Petroica boodang* (Lesson, 1838) should now be universally adopted for Scarlet Robin populations on mainland Australia (sensu Schodde and Mason 1999), which have long been thought to belong to *P. multicolor* (Mayr 1934; Boles 2007; BirdLife International 2012).

Our analyses also suggest that a case could be made for the description of the Solomon Islands and Vanuatu/Fiji/Samoa lineages of Pacific Island Robins as separate species under the same criteria. If so, *Petroica pusilla* Peale, 1848 has priority for the lineage in Vanuatu, Fiji and Samoa. The Solomon Islands lineage, however, has three valid and

available species-group epithets, each of which was erected by Mayr (1934). The epithets are *polymorpha* Mayr, 1934, *kulumbangrae* Mayr, 1934, and *septentrionalis* Mayr, 1934. Under Article 24.2.1 of the International Code of Zoological Nomenclature (ICZN (International Code of Zoological Nomenclature) 1999) we as first reviser nominate *polymorpha* Mayr, 1934, meaning 'many forms', to apply as a species rank epithet for the Solomon Islands lineage. However, this hypothesis will need to be evaluated more fully elsewhere using increased sampling of nuclear loci. At the very least, it is clear from our data that these two lineages should be recognised as distinct evolutionary significant units (ESUs) (Moritz 1994).

Diversification of *Petroica*

Here, we present the first phylogeny of *Petroica* to include complete taxon sampling of the iconic Pacific robin radiation (*P. multicolor* species complex). We combine these Pacific robin samples with all recognized species of *Petroica* in Australia and one representative from the New Zealand clade (*P. macrocephala*). Note that the monophyly of the four species in New Zealand (*P. macrocephala, P. australis, P. longipes, P. traversi*) with respect to Pacific Island Robins and Norfolk Island Robins was previously established by Miller and Lambert (2006) using mitochondrial control region and cytochrome b. Although Miller and Lambert (2006) did not include any Australian species, the representatives of the New Zealand clade used in our study (*P. macrocephala*) and Christidis et al. (2011) (*P. macrocephala, P. australis*) are consistently placed separate from Australian species in phylogenetic analyses of two other mitochondrial loci (*ND2, CO1*) and three nuclear introns (*beta-fibrinogen intron 5, ACO1 intron 9, CLOCK intron 10*) (Figs. 2, 3; Christidis et al. 2011).

Together with previous studies our analyses offer support for the division of *Petroica* into three main clades: (1) Australian clade (*P. boodang, P. rosea, P. rodinogaster, P. phoenicea*), (2) New Zealand clade (*P. macrocephala, P. australis, P. longipes, P. traversi*) and (3) Pacific clade (Norfolk Island, Pacific Island and Red-capped Robins) (Miller and Lambert 2006; Loynes et al. 2009; Christidis et al. 2011; Figs. 2, 3, this study). Thus there is now strong evidence against the long-standing hypothesis (Boles 2007) that Pacific Robins form a species-group with the New Zealand Tomtit *P. macrocephala* and Scarlet Robins *P. boodang* (Miller and Lambert 2006; Christidis et al. 2011; this study). However, the details of relationships between and within the three major clades remain uncertain owing to poor support at deeper nodes in the phylogeny (Miller and Lambert 2006; Loynes et al. 2009; Christidis et al. 2011; Figs. 2, 3, this study), as well as the lack of samples from two species in New Guinea (*P. archboldi* and *P. bivittata*). Dense taxon sampling and multiple nuclear loci are needed to provide a more complete picture of relationships and diversification across all *Petroica*.

Discriminating between alternative biogeographic hypotheses and determining the timing and origin of different Pacific colonizations also awaits denser locus and taxon sampling. We have clear support for the diversification of two distinct *Petroica* lineages in the southwest Pacific—one centered in New Zealand and one centered in the Pacific archipelagos (Miller and Lambert 2006; Christidis et al. 2011; Figs. 2, 3, this study). However, it remains unclear whether these result from "upstream" or "downstream" colonizations. For example, did Norfolk and Pacific Island Robins originate from two separate "downstream" colonizations of the southwest Pacific from an Australian (or New Guinean) ancestor or did Red-capped Robins originate from reverse "upstream" colonization of mainland Australia from a southwest Pacific ancestor (e.g., Clegg et al. 2002; Filardi and Moyle 2005; Jones and Kennedy 2008; Cibois et al. 2011; Andersen et al. 2013)?

Disparate biogeographic histories on Norfolk Island

Our finding of a close phylogenetic relationship between Norfolk Island Robins and Australian Red-capped Robins to the exclusion of the New Zealand *Petroica* robins contrasts with the biogeographic histories of other Norfolk Island fauna. For example, phylogenetic studies have shown that the Norfolk Island Pigeons (*Hemiphaga novaeseelandiae spadicea*), Norfolk Boobooks, Norfolk Parakeets and Norfolk Silvereyes (*Zosterops lateralis lateralis*) are all most closely related to their congenerics/conspecifics in New Zealand, Lord Howe Island and New Caledonia rather than those in Australia (Norman et al. 1998; Boon et al. 2001; Estoup and Clegg 2003; Goldberg et al. 2011). Finding such disparate biogeographic histories on Norfolk Island is not surprising given that the overall distributions of the taxa studied to date differ substantially (Schodde et al. 1983; Keast 1996; Keast and Miller 1996; Trewick and Gibb 2010). Thus, our study reinforces the hypothesis that Norfolk Island harbors biota of diverse biogeographic origins and histories. This highlights the necessity to conservation efforts of characterizing the taxonomic distinctiveness and phylogenetic relationships of Norfolk Island's threatened fauna (Garnett et al. 2003). Such efforts have been hindered by the limited availability of contemporary tissues from threatened Norfolk Island taxa. Our study clearly shows that in the absence of contemporary tissue samples, ancient DNA approaches using historical museum specimens can offer strong support for taxonomic hypotheses that morphological traits alone are unable to resolve. Similar ancient DNA-based analyses should now be applied to other

threatened fauna in Norfolk Island with uncertain taxonomic distinctiveness and phylogenetic affinities (e.g., Norfolk Golden Whistlers: Commonwealth of Australia 2005; Director of National Parks 2010; Andersen et al. 2014). This is especially important given the increasing threat of climate change, habitat loss and predation to island populations in the southwest Pacific Ocean (Kingsford et al. 2009; Keppel et al. 2012; Şekercioğlu et al. 2012), including Norfolk Island (Director of National Parks 2010; Dutson 2013).

Conclusion

We have presented clear evidence that the Norfolk Island Robin is both phenotypically and genetically distinct from other taxa, and that it should be recognized as a distinct species. This taxonomic change will require a reevaluation of its conservation status and management plan (Director of National Parks 2010; Garnett et al. 2011b; Dutson 2013). Despite being significantly threatened (see Introduction), Norfolk Island Robins are listed as 'Least Concern' by the IUCN (BirdLife International 2012). This is because the IUCN Red List is primarily focused at the species-level and the current assessment for Norfolk Island Robins follows the taxonomy that combines Norfolk Island Robins as a weakly differentiated subspecies of a widespread species that includes Scarlet Robins and Pacific Island Robins (following Mayr 1934; Higgins and Peter 2002; Boles 2007; BirdLife International 2012). We have shown that this is unrepresentative of both the taxonomic and conservation status of the Norfolk Island Robin. Accordingly, we propose that the Norfolk Island Robin be added to the IUCN Red List as an endemic endangered species.

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Appendix IV

A case for realigning species limits in the southern Australian whipbirds long recognized as the Western Whipbird *Psophodes nigrogularis*

This appendix contains the publication of research investigating the taxonomy of southern Australian whipbirds. I contributed to this work by performing the DNA extraction and sequencing of historical museum specimens. This article has been published in *Emu.*

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A case for realigning species limits in the southern Australian whipbirds long recognised as the Western Whipbird (*Psophodes nigrogularis*)

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ABSTRACT

The Western Whipbird (*Psophodes nigrogularis*) has a highly disjunct west–east distribution across southern Australia. Earlier morphological analyses recognised four subspecies in one species: *P. n. nigrogularis* and *P. n. oberon* in south-west Western Australia, and *P. n. leucogaster* of the Eyre and Yorke Peninsulas and the Murray Mallee, and *P. n. lashmari*, restricted to Kangaroo Island, both in eastern Australia. Later morphological analyses elevated *P. n. nigrogularis* to monotypic species rank, and placed the remaining western and two eastern taxa as three subspecies of a second species *P. leucogaster*. Initial mtDNA analysis questioned both arrangements but could not include all taxa. We used mtDNA sequence data from all available specimens of the entire group (DNA extracted from cryo-preserved tissues, toe-pads and feathers; holotypes excepted) to derive a more stable view of species limits. The samples fell into two strongly divergent but geographically structured groups, completely reflecting the eastern and western components of the distribution. Thus we see merit in treating the two geographical groupings as two species, *P. nigrogularis* in the west and *P. leucogaster* in the east, each having two subspecies. Nuclear data could test whether the two western subspecies in particular are genetically isolated or currently exchanging genes.

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Introduction

The whipbirds and wedgebills of Australia and New Guinea are a small group of oscine passerines that are part of the Corvoidea. Conventionally, they are assigned to two genera (*Psophodes* having four or five Australian species, and *Androphobus* having one species, the New Guinean Whipbird (*A. viridis*)) placed in the Psophodidae, a family of long-uncertain limits and relationships. The most recent reviews of the group's family-level relationships have restricted Psophodidae to include only *Psophodes* and *Androphobus* and suggested their closest living relatives include the Vireonidae of the Americas as well as other Australo-Papuan groups (Norman *et al.* 2009; Aggerbeck *et al.* 2014; see also Hugall and Stuart-Fox 2012).

Psophodes is usually recognised as including the Chirruping and Chiming Wedgebills, *P. cristatus* and *P. occidentalis*, respectively, of the Australian arid zone, and the Eastern and Western Whipbirds, *P. olivaceus* and *P. nigrogularis*, respectively, of wetter habitats in eastern and southern Australia. Debate has arisen, however, as to whether the Western Whipbird, having a highly disjunct west–east distribution across southern Australian mallee heaths (south-west Western Australia in the west and the Eyre Peninsula–Kangaroo Island–Yorke Peninsula–Murray Mallee region of South Australia and western Victoria in the east; Figure 1), comprises one or two species. After reviewing geographical variation in Western Whipbird morphology based on the 23 specimens available, Schodde and Mason (1991) recognised two subspecies in south-west Western Australia, *P. n. nigrogularis* and *P. n. oberon*, noting that their ranges 'evidently abut (or have abutted) on one another immediately north and east of King George Sound–Two Peoples Bay in Western Australia, raising questions about their taxonomic status' (p. 139). In eastern Australia, they recognised a further two subspecies, *P. n. leucogaster* of the Eyre and Yorke Peninsulas and the

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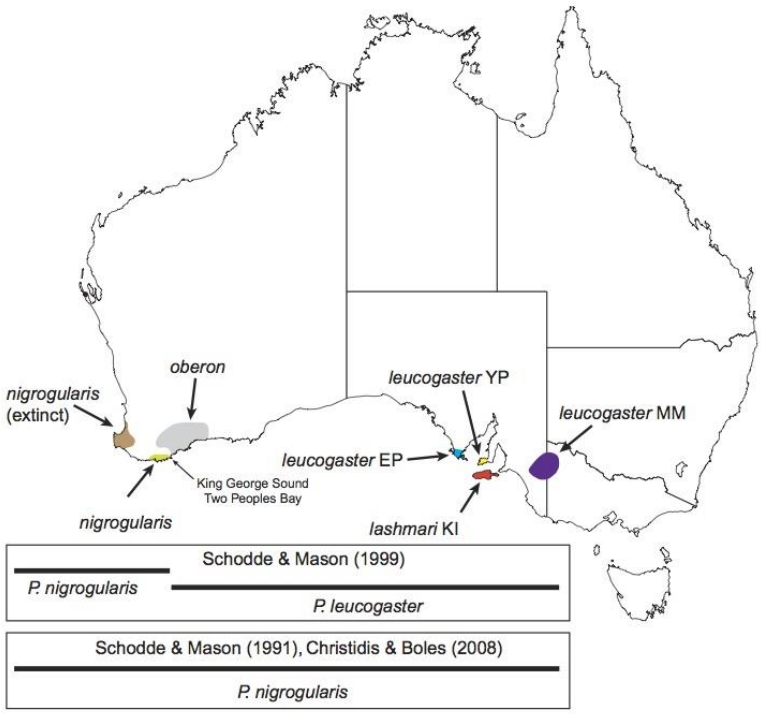

Figure 1. Current distribution of four recognised taxa (*nigrogularis, oberon, leucogaster* and *lashmari*) within Western Whipbirds (*Psophodes nigrogularis sensu lato*) across southern Australia. YP = Yorke Peninsula, EP = Eyre Peninsula, MM = Murray Mallee, KI = Kangaroo Island). Taxonomic treatments according to the one- or two-species hypotheses described in the text are summarised at the bottom of the figure.

Murray Mallee, and *P. n. lashmari*, restricted to Kangaroo Island. Importantly, they noted that these four forms fell into two groups. One was represented only by nominotypical *P. n. nigrogularis*, and was relatively small, plain olive-grey with dull-banded tails, and tended to be sexually dimorphic on the belly. The other three subspecies were larger, greyer and whiter-bellied birds with brightly banded tails (see Schodde and Mason 1999 for reassessment and expansion of the differences).

Schodde and Mason (1999) later argued that these two groups should indeed be recognised as two species. They thus restricted the Western Whipbird (*P. nigrogularis*) to what had been recognised as *P. n. nigrogularis* and assigned the other three taxa to the Mallee Whipbird (*P. leucogaster*) (Figure 1). Christidis and Boles (2008) and later electronic lists (e.g. BirdLife International 2014; Gill and Donsker 2014) recognised only one species, the Western Whipbird (*P. nigrogularis*), for all four forms.

Toon *et al.* (2013) presented a phylogenetic overview of relationships among all Australian *Psophodes* based on the mitochondrial DNA (mtDNA) ND2 gene and focused primarily on placing the diversity in their vocalisations in a phylogenetic context. Although they were unable to sample all populations just discussed, they found that samples of the two western taxa *oberon* and *nigrogularis* were more closely related to each other than either was to eastern *lashmari* (Figure 2) and they recorded ~2.2% net divergence between the taxa *lashmari* and *nigrogularis*. This result is at least strongly suggestive that a third hypothesis might usefully be considered, namely that all Western Australian populations comprise one species and the eastern Australian ones comprise another. The aim of this paper, then, is to use DNA sequence data derived from the few available cryo-preserved samples as well as from DNA extracted from toe-pads and feathers of specimens representing all four taxa in an attempt to derive a more stable view of relationships and thus where species limits might be drawn within the Western Whipbird (*P. nigrogularis sensu lato*) group. We acknowledge that the nature of the samples has dictated that our analyses were most readily confined to mitochondrial DNA (mtDNA); we focus

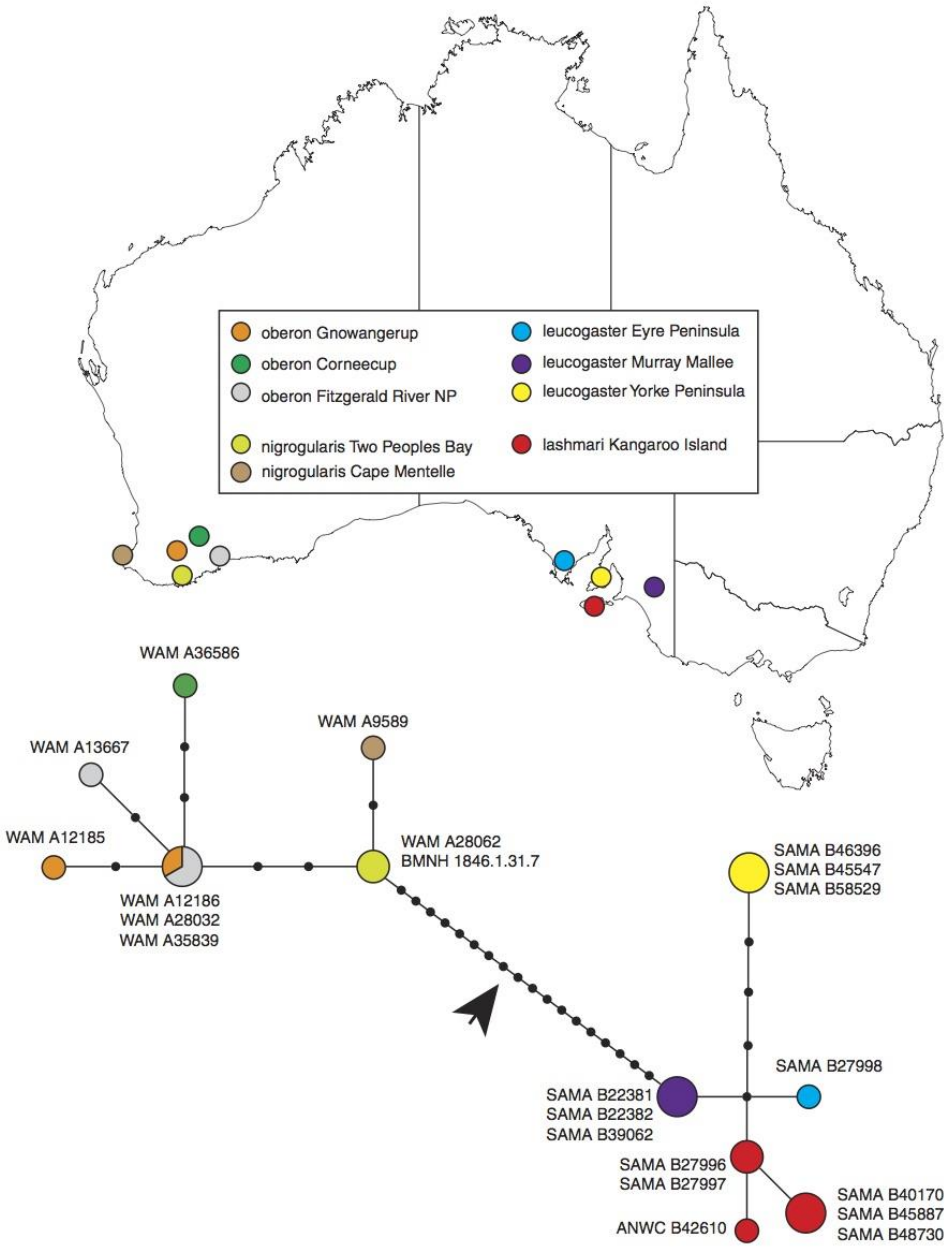

Figure 2. Haplotype network for 1114 bp of mtDNA ND2 sequence from 22 Western Whipbird samples from each of the four currently recognised taxa (*nigrogularis, oberon, leucogaster* and *lashmari*) across southern Australia. Individual haplotypes are coloured by sampling location and taxonomic assignment, with museum accession numbers for each specimen. Small black dots represent unobserved haplotype and each line segment represents a single nucleotide substitution. The large arrow indicates the position of the root of the network in the maximum parsimony analysis.

on the ND2 gene, which is commonly used in species-level systematics in birds. For brevity and unless indicated otherwise, we use the four species/subspecies epithets *nigrogularis*, *oberon*, *lashmari* and *leucogaster* to refer to the populations indicated in Figure 1.

Materials and methods

Samples

We included three contemporary (blood/tissue) and 20 historical (toe-pads/feathers) museum samples from all four currently recognised subspecific taxa of the *P. nigrogularis* group (Figure 1; Table 1). Mitochondrial ND2 sequences from the three contemporary samples were generated in a previous study (Table 1; Toon *et al.* 2013), while the 20 historical samples were newly analysed. Three specimens of *nigrogularis* were available, including one from its present range and two from the extinct population in the Cape Mentelle region further west. Nineteen specimens (6–7 samples per taxon) of the remaining three subspecies were sampled.

Molecular analysis

DNA extraction, mtDNA ND2 PCR amplification and DNA sequencing of the three contemporary specimens is described in Toon *et al.* (2013). Contamination of the historic museum samples with contemporary DNA and previously amplified mtDNA PCR products was controlled by conducting all pre-PCR work in a dedicated and physically separate clean-room DNA facility at the Australian Centre for Ancient DNA, University of Adelaide. No contemporary bird samples or DNA had ever been present in the pre-PCR laboratory. The pre-PCR laboratory included the use of separate dead-air glove boxes for DNA extraction and PCR set-up, regular decontamination of all work areas and equipment with sodium hypochlorite, personal protective equipment (PPE) including disposable laboratory gown, face mask, hair net, face shield, shoe covers and double-gloving and strict one-way movement of personnel.

Sampling restrictions and methodology aimed at minimising destructive sampling of museum specimens limited toe-pad/feather sample size to enough for just a single DNA extraction attempt for all historical museum specimens. DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions with the following modifications, which have proven useful in other studies (e.g. Austin *et al.* 2013): (i) 12 µl of 1 M DTT was added to each sample before proteinase K digestion, (ii) 2 µl of carrier RNA was added to each sample following the addition of ethanol, and (iii) DNA was eluted twice from the silica spin columns with 50 µl of buffer AE for a final volume of 100 µl. A negative extraction control was included to monitor for contamination.

The mtDNA ND2 gene was amplified using six sets of primers targeting 194–264 base pair (bp) overlapping fragments. Primer combinations were: fragment 1 (5′-GGCCCATACCCCGRAAATG-3′/5′-GGGGGTGGTGGGATTTTGAG-3′), fragment 2 (5′-TGAGTTATAGCTTGAACCGG-3′/5′-GAAGTGGAA TGGCACTAGTC-3′), fragment 3 (5′-TATCACACA ACTAACCCACC-3′/5′-GCCCTATTCATCCCCCTA G-3′), fragment 4 (5′-TCAACACTGCTAACAAC CAT-3′/5′-TCATGCAGTTATTAGGGCTG-3′), fragment 5 (5′-ACAATCAAAGTCCTAAAACT-3′/5′-GTGTAATTGTTGCACAGTATG-3′) and fragment 6 (5′-CCCTGCTAGGGCTATTCTTCT-3′/5′-ACTCTT RTTTAAGGCTTTGAAGGC-3′). PCRs were performed in 25 µl volumes containing 2 µl of extracted DNA, 1× High Fidelity PCR Buffer (Invitrogen, California, USA), 200 µM of each dNTP, 400 µM of each primer (Geneworks, Thebarton, South Australia), 2 mM $MgSO_4$ 1 µg/µl RSA (Sigma, Darmstadt, Germany) and 0.5 units of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, California, USA). Thermocycling conditions were: denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 30 s, with a final extension step at 68°C for 10 min. All PCR attempts included a PCR no template control and the relevant negative extraction controls for the samples being amplified.

PCR products were visualised under UV light on 2–3.5% agarose gels stained with GelRed (Biotium Incorporated, Hayward, California, USA). Successful amplifications were sent to the Australian Genome Research Facility (Adelaide) for Sanger Sequencing and capillary electrophoresis. Sequence chromatograms were edited and assembled using Geneious 7.1.2 (Biomatters, Auckland, New Zealand).

Data analysis

DNA sequences were aligned using Geneious 7.1.2 (Biomatters). TCS 1.2 (Clement *et al.* 2000) was used to calculate haplotype parsimony networks using a 90% connection limit. To test for monophyly of eastern and western birds, we estimated the position of the root on the network using maximum parsimony searches in PAUP* (Swofford 2002) with a heuristic search and TBR branch swapping of 1000 replicates with random sequence addition. The three other recognised species of *Psophodes* whipbirds and wedgebills (Toon *et al.* 2013) were included as outgroups. Analyses were run with and without individuals with missing data. DnaSP 5.10 (Librado and Rozas 2009) was used to measure nucleotide and haplotype diversity and the net nucleotide divergence between taxa and regions. A hierarchical analysis of molecular variance (AMOVA) was

Table 1. Specimen details for *Psophodes nigrogularis sensu lato* samples included in this study

Species	Subspecies	Distribution	Museum accession number	Sample type	Locality	GenBank accession
P. nigrogularis	*nigrogularis*	SW Western Australia	WAM A28062	Tissue/blood	Two Peoples Bay, WA	KC821981[a]
			WAM A9589	Toe-pad	Cape Mentelle, WA	KY929519
			AMNH 458994	Toe-pad	Cape Mentelle, WA	no DNA obtained
	oberon	SW Western Australia	WAM A12815	Toe-pad	Gnowangerup, WA	KY929517
			WAM A12816	Toe-pad	Gnowangerup, WA	KY929514
			WAM A13667	Toe-pad	Fitzgerald River National Park, WA	KY929518
			WAM A28032	Toe-pad	Fitzgerald River National Park, WA	KY929515
			WAM A35839	Toe-pad	Fitzgerald River National Park, WA	KY929516
			WAM A36586	Tissue/blood	Corneecup Nature Reserve, WA	KC821980[a]
	Unknown	Western Australia	NHMUK 46.1.31.7	Toe-pad	Unknown	KY929520
	lashmari	Kangaroo Island	ANWC B42610	Tissue/blood	Kangaroo Island, SA	KC821979[a]
			SAMA B40170	Toe-pad	Kangaroo Island, SA	KY929506
			SAMA B45887	Toe-pad	Kangaroo Island, SA	KY929504
			SAMA B27997	Toe-pad	Kangaroo Island, SA	KY929502
			SAMA B27996	Toe-pad	Kangaroo Island, SA	KY929503
			SAMA B48730	Toe-pad	Kangaroo Island, SA	KY929505
	leucogaster	Southern South Australia	SAMA B22381	Toe-pad	Pinnaroo, Murray Mallee, SA	KY929507
			SAMA B22382	Toe-pad	Pinnaroo, Murray Mallee, SA	KY929509
			SAMA B39062	Toe-pad	Malinong district, Murray Mallee, SA	KY929508
			SAMA B46396	Toe-pad	Yorke Peninsula, SA	KY929511
			SAMA B55457	Feather	Yorke Peninsula, SA	KY929513
			SAMA B58529	Feather	Yorke Peninsula, SA	KY929512
			SAMA B27998	Toe-pad	Eyre Peninsula, SA	KY929510

[a] Sequences from Toon *et al*. (2013).

Abbreviations used: SW – south-western; WA – Western Australia; SA – South Australia; WAM – Western Australian Museum, Perth; ANWC – Australian National Wildlife Collection, Canberra; SAMA – South Australian Museum, Adelaide; AMNH – American Museum of Natural History, New York; NHMUK – Natural History Museum, Tring.

conducted in ARLEQUIN 3.5.2 (Excoffier and Lischer 2010) to assess partitioning of mtDNA diversity within vs. between subspecies under the assumption of a single species or two species (western and eastern) each containing two subspecies.

Morphological analysis

In order to assess geographic variation in morphological characters we used measurements provided by Schodde and Mason (1991) together with measurements from another two more recently collected specimens held in the Western Australian Museum. The two new specimens were both referable to *oberon* – one was from Jacup (Fitzgerald River National Park) and the other from Corneecup Nature Reserve, near Nyabing. Measurements (wing, tail, bill, tarsus, tail bar; see Schodde and Mason (1991) for details of methods) were available for 16 individuals once juveniles and specimens with missing data were removed. We used ordination by multidimensional scaling (MDS) from the program Primer v6 (Clarke and Gorley 2006) to reveal patterns of morphological similarity in the data matrix. All data were normalised, and the Euclidean resemblance measure was used to compare the specimens according to their morphometric similarities.

Diagnosis of species limits

We follow the species concept outlined by Gill (2014). The biological species concept (Mayr 1942) is an unsatisfactory framework for assessing the taxonomic status of allopatric populations for reasons outlined by many, but most recently Gill (2014). Further, the formulaic approach adopted by Tobias *et al.* (2010) and del Hoyo *et al.* (2014) cannot accommodate differing rates of evolution of morphological and molecular traits and we simply are suspicious of any formulaic approach to describing evolution taxonomically. In the absence of nuclear DNA markers indicative of male gene flow, we examine the phylogenetic structure of mtDNA gene trees and patterns of divergence among different populations of Western Whipbirds *sensu lato*.

Results

We obtained 1014 bp of mtDNA ND2 sequence from 20 of 23 samples of Western Whipbirds (*P. nigrogularis sensu lato*). One sample, NHMUK 46.1.31.7 (see Table 1 and its footnote for museum acronyms), yielded only 654 bp of sequence while a second sample, WAM A9589, yielded 863 bp of sequence. A third sample, AMNH 458994, which was one of two of the extinct Cape Mentelle *nigrogularis* population, failed to yield amplifiable DNA. Thirty-nine polymorphic sites defined 12 unique haplotypes each represented by up to three individuals. Individual haplotypes differed by 1–27 nucleotide substitutions.

The 22 samples fell into two groups separated by 17 fixed differences (1.7% net nucleotide divergence); the two groups reflected the eastern and western parts of the geographical range (Figures 1 and 2). The root fell between the eastern and the western haplotype groups, supporting that the eastern and western birds are reciprocally monophyletic (Figure 2). Haplotype and nucleotide diversity in the eastern and western groups was similar, namely 0.872, 0.889 and 0.00273, 0.00278, respectively. Six of the western samples were *oberon* (haplotype diversity = 0.800, nucleotide diversity = 0.00234). Among these, one haplotype was shared among three individuals, the three other being singletons each no more than three nucleotide differences divergent from the shared haplotype. The remaining three western samples were *nigrogularis* (WAM 28062, Two Peoples Bay, WAM A9589 Cape Mentelle and BMNH 1846.1.31.7). We note that NHMUK 1846.1.37.1 had no locality data but had been tentatively assigned to *nigrogularis* by R. Johnstone (pers. comm.), and the DNA data supported this. The three *nigrogularis* were separated from the shared *oberon* haplotype by three to five nucleotide differences. Among the eastern samples, six haplotypes were found among the 13 sampled individuals. The six Kangaroo Island individuals (*lashmari*) had three haplotypes no greater than two nucleotide differences from each other (haplotype diversity = 0.733, nucleotide diversity = 0.00092). Next, the seven samples from the three sampled regions of the range of *leucogaster* (haplotype diversity = 0.714, nucleotide diversity = 0.00254) comprised three haplotypes, one each from the Murray Mallee (three individuals) and the Yorke Peninsula (three individuals) being unique to those areas and five nucleotides divergent from each other. Finally, the remaining, single sample from Eyre Peninsula was two and five nucleotides divergent, respectively, from the Murray Mallee and Yorke Peninsula samples, and two or three nucleotides divergent from Kangaroo Island samples.

The results of the AMOVA revealed that mtDNA diversity was partitioned predominantly among the four subspecies (87.4%) rather than within subspecies (12.6%), but that this is driven largely by the east–west phylogenetic split (78.5% of the variation between eastern and western groups), with only 12.2% between subspecies within each region and 9.2% within subspecies.

The four currently recognised subspecies also separated out on the ordination plot (Figure 3) but there is no compelling morphological evidence in this analysis for the separation of these taxa into two groups.

Discussion

We set out to determine whether patterns of mtDNA diversity could inform the questions of whether the Western Whipbird (*Psophodes nigrogularis sensu lato*) of southern Australia comprises one or two species (Schodde and Mason 1991, 1999) and where species limits can best be drawn. A critical point to test was whether the two taxa recognised in south-western Western Australia, *nigrogularis* and *oberon*, were closest relatives. This had been suggested by an earlier molecular study (Toon *et al.* 2013) that was unable, however, to include all relevant taxa. The alternative was that one of them, *oberon*, was more closely related to eastern Australian *lashmari* and *leucogaster* as Schodde and Mason's (1999) formulation of a two-species hypothesis predicted. Our first key finding was that the samples fell into two strongly divergent geographically structured groups, which completely reflected the eastern and western components of the distribution. This strikingly parallels what Murphy *et al.* (2011) found in the ecologically similar Eastern and Western Ground Parrots (*Pezoporus wallicus* and *P. flaviventris*, respectively). It reinforced the earlier finding by Toon *et al.* (2013) that *nigrogularis* and *oberon* are more closely related to each other than either is to the other forms. Our second key finding is that the level of genetic divergence between our samples of *nigrogularis* and *oberon* closely approximates that between the three samples of the Yorke Peninsula population of *leucogaster* and all other eastern samples of that taxon and *lashmari*. We now discuss these findings in more detail.

The most important taxonomic aspect of our findings, then, is that *nigrogularis* being one species and all other populations, including western *oberon*, being a second species (*sensu* Schodde and Mason 1999) finds little support in our molecular data or morphological analysis, though this does not in itself reject Schodde and Mason's (1999) descriptions of morphological diversity. Aiming to derive a testable taxonomic hypothesis that reconciles patterns of morphological and molecular diversity in the group, we suggest several other taxonomic possibilities. One is simply to recognise a single species, *P. nigrogularis*, having up to four subspecies based on current taxonomy, two in Western Australia and two in eastern Australia. Notably, however, the 17 fixed differences between eastern and western groups translate to a net divergence from each other of 1.7%, which is towards the higher end of intraspecific divergences empirically observed in mtDNA (e.g. Avise and Walker 1998 for early mtDNA data; Hebert *et al.* 2004 for North American birds; Weir 2006 for neotropical birds; Joseph and Omland 2009 for Australian birds). This pattern of slight morphological differentiation coupled with varying levels of mtDNA divergence parallels other cases of southern Australian birds having similarly isolated populations in eastern and western

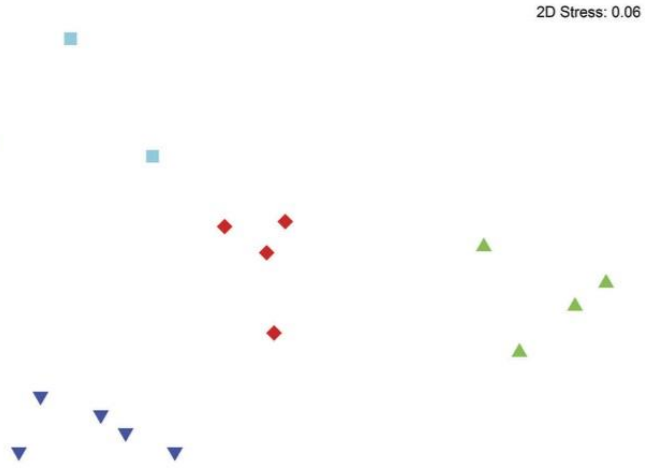

Figure 3. Ordination (MDS) plot of normalised morphometric data for the four currently recognised taxa in the *Psophodes nigrogularis* group (triangles = *nigrogularis*, inverted triangles = *oberon*, diamonds = *leucogaster* and squares = *lashmari*). The *leucogaster* group contains three specimens from north-west Victoria and eastern South Australia, and one from Yorke Peninsula.

Australia. Cases involving species inhabiting similarly shrubby or sclerophyllous heath-like habitats as the whipbirds, or indeed chenopodiaceous steppe habitats, have been described by Murphy *et al.* (2011), Dolman and Joseph (2012, 2015) and Austin *et al.* (2013). Our findings are not readily predicted by a one-species/four-subspecies hypothesis or by a two-species hypothesis where *nigrogularis* is one monotypic species and *oberon*, *lashmari* and *leucogaster* are the other species. Further, we would argue that a one-species hypothesis might be challenged to address what kind of data would be needed over and above that which we have reported here to reject a one-species hypothesis.

We suggest that the new data shift the balance of focus for future work in favour of a two-species hypothesis by which the two geographical groupings are treated as two species, each having two subspecies. Nuclear data could most importantly be used to determine whether the two subspecies of the western species in particular are genetically isolated or currently exchanging genes. Eastern and western species as so construed will undoubtedly be found to share diversity at many nuclear DNA loci. Given the obvious geographical isolation of eastern and western populations from each other, this would most likely be due to eastern and western populations retaining ancestral variation rather than due to gene flow between them. Shared ancestral variation for nuclear genes is routinely observed at this taxonomic level and reflects nDNA's often slower rate of evolution than that of mtDNA (e.g. reviews in Beheregaray 2008; Joseph and Omland 2009; Edwards *et al.* 2016). Further, as whipbirds are sedentary and have low capacity for dispersal (reviewed in Higgins and Peter 2002), gene flow is inherently unlikely between eastern and western Australian populations. In addition, preliminary observations on variation in vocalisations (reviewed in Higgins and Peter 2002; see also McGuire 2011 and supplementary material herewith) show that there is considerable variation within and between individuals as well as variation between locations; this deserves further exploration but detailed analysis of the extent of variation within and between populations is beyond the scope of the present study. In one study (McGuire 2011), which included one population of *nigrogularis* and two of *leucogaster*, eastern females did not respond to playback of western males but males did, suggesting that there may be some level of a pre-mating isolation mechanism in place. Interestingly, female songs also differed geographically and – similar to the male song playback experiments – eastern females were more discerning than males, responding only to eastern female songs (McGuire 2011). These differences are consistent with the remarkably different patterns of geographic variation between the sexes of the closely related Eastern Whipbird (*P. olivaceus*), in which there is little geographic variation in male calls but pronounced differences in female calls at a continental scale (Mennill and Rogers 2006).

The two-species hypothesis we suggest, while consistent with the phylogenetic analysis of Toon *et al.* (2013), is perhaps less consistent with described patterns of morphological diversity. The latter may indicate shared ancestral states among *oberon* and eastern populations rather than close relationship. Perhaps most importantly, we would caution that if *nigrogularis* alone were recognised as a species on the basis of its greater mtDNA divergence within western populations, then the comparable divergence of the Yorke Peninsula population from other eastern populations might also be argued to warrant its species-level recognition. This seems premature at present but an epithet, *pondalowiensis*, is available for the Yorke Peninsula birds (Condon 1966). The holotype of the name *P. leucogaster* Howe and Ross, 1933 is from the Murray Mallee population so by that arrangement Kangaroo Island and Murray Mallee populations would remain within *P. leucogaster*.

Schodde and Mason (1991, 1999) have drawn attention to concerns that Two Peoples Bay is a locality within the range of *nigrogularis*. Our assignment and that of Toon *et al.* (2013) of the single specimen we have from that locality of *nigrogularis* is based on its being a specimen one of us (A.H.B.) has examined in the Western Australian Museum (WAM 28062) and its clear divergence from samples of the other taxa. What our analyses based on limited sample sizes still cannot reject is that there is a more complex genetic interaction among the Western Australian populations.

In conclusion, we recommend that the best available taxonomic hypothesis for these birds and the one that future research might attempt to reject is that of recognition of two species, *P. nigrogularis* in south-western Australia and *P. leucogaster* in eastern Australia, each with two subspecies *P. n. nigrogularis* and *P. n. oberon*, and *P. l. leucogaster* and *P. l. lashmari*. We consider this to be the taxonomic hypothesis that best describes the group's history and which is most readily testable through more extensive sampling of genes and individuals. Detailed consideration of English names is beyond our scope but we advocate no further use of the names Western Whipbird or Mallee Whipbird. Perhaps Black-throated Whipbird for *P. nigrogularis* and White-bellied Whipbird for *P. leucogaster* could be considered. Our data suggest the need to reconcile discordant patterns of morphological and molecular diversity in the birds,

particularly if the western population assigned to *nigrogularis* is as morphologically distinct as Schodde and Mason (1999) noted. If so, that would open the question as to which selective agents might be causing that discordance. Closer study is warranted of within-population-level diversities. Given the rarity of the birds, this should be done through larger non-destructively obtained sample sizes and with newer genomics-based methods. Also needing study is whether there is gene flow among the western populations, and, finally, the suggestion in our data that the Yorke Peninsula population warrants recognition with the epithet *pondalowiensis*, being more divergent than might have been appreciated until now.

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