Population Genetics and Phylogeography of Antilopine Wallaroo Using Degraded DNA from Scats and Museum Specimens

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Thesis abstract

The research conducted in this thesis utilises faecal pellets (scats) and museum samples to examine the population genetics of one of Australia's large macropods, the antilopine wallaroo, *Macropus antilopinus*, across the monsoonal tropics of northern Australia. The project focussed on populations of antilopine wallaroos and other large sympatric macropods in north-eastern Australia where visual and indirect (scat) surveys were undertaken at 53 sites. Macropod scats were collected from transect points at the same sites where the visual surveys were conducted.

Macropod scats are often difficult to identify based only on morphology. Prior to their use in species occurrence and population genetic analyses a simple, rapid DNA-based identification test was developed to positively identify scats from seven species of *Macropus* that occur in north-eastern Australia. The identified scats were then included in an indirect genetic survey to examine their utility as an alternative to visual surveys to determine species occurrence. The presence and absence data from the survey was used to investigate the ecological determinants of occurrence for each species. The indirect, molecular derived occurrence data, alone, can be used to develop informative ecological models that describe the inter-specific habitat requirements of macropods. The indirect genetic survey of macropods was also cheaper and less time consuming to conduct, and provided more occurrence records (and less false absences), then visual surveys.

The non-invasively collected scats also provide an excellent source of genetic material for further genetic analysis including determining the patterns of dispersal and contemporary population structure. Scats from two populations of antilopine wallaroo from the latitudinal extremes of the Cape York distribution were analysed using 15 microsatellite loci. Genetic diversity within the two populations was low compared to the levels reported in other macropod species. Population structure analyses indicated that gene flow between the two populations was restricted. Population structure and the current pattern of gene flow within antilopine wallaroo were examined further to elucidate phylogeographic structure within the species, and explore potential causes of geographic variation from the species entire range. Museum samples, including bone and skin, from the remainder of the species distribution were utilised to determine the historical aspects of the contemporary spatial distributions of gene lineages across the species range. Evidence for significant phylogeographic structuring

across northern Australia was found and can be related to know biogeographical barriers in the area. Divergence dates calculated for the major mtDNA clades suggested the environment and climate changes associated with glacial cycles may have facilitated this diversification.

The thesis demonstrates some of the many applications of utilising non-invasive sampling for both ecological and evolutionary studies of species. Once an accurate identification of species is made, occurrence, distribution, environmental and genetic data can all be used to create better informed management decisions for antilopine wallaroo.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other 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 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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Jessica J Wadley

December 2013

Publications

The publications emanating from the period of PhD candidature, that have been published, submitted or are currently in preparation, include:

Chapter II – (Published) Wadley JJ, Austin JJ, Fordham DA (2013) Rapid species identification of eight sympatric northern Australian macropods from faecal-pellet DNA. *Wildlife Research* **40** (3), 241-249.

Chapter III – (Submitted to Austral Ecology) Wadley JJ, Austin JJ, Fordham DA (2013) Genetic inference as a method for modelling occurrence: a viable alternative to visual surveys.

Chapter IV – (Published) Wadley JJ, Austin JJ, Gardner MG, Fordham DA (2013) Fifteen microsatellite loci for use in non-invasive sampling studies of antilopine wallaroo (*Macropus antilopinus*). *Australian Journal of Zoology*, online early

Chapter V – (In prep for Ecology and Evolution) Wadley JJ, Fordham DA, Austin JJ (2013) Low genetic diversity and limited population structure in a large, vagile macropod (antilopine wallaroo; *Macropus antilopinus*) from Australia's tropical savannahs.

Chapter VI – (In prep for Journal of Zoology) Wadley JJ, Thomson VA, Fordham DA, Austin JJ (2013) Phylogeography of the antilopine wallaroo (*Macropus antilopinus*) across northern Australia.

Appendix II – (Published) Wadley JJ, Austin JJ, Ritchie EG, Fordham DA (2013) The importance of knowing your roo poo. e-Science, issue 7, October 2013 – Research byte pages 16-17.

Appendix III – (Published) Burridge CP, Brown B, Wadley J, Nankervis D, Olivier L, Hull C, Barbour R, Austin JJ (2013) Did post-glacial changes in sea-level initiate the evolutionary divergence of a Tasmanian 'endemic' raptor from its mainland relative? *Proceedings of the Royal Society B: Biological Sciences* **280** (1773), online early.

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Chapter I: General introduction

Kangaroo Island western grey kangaroo ©Jessica Wadley

Chapter I: General introduction

Biodiversity loss is a global issue with significant ecological and economic impacts. Worldwide, human populations are growing rapidly and resources are being consumed at unprecedented rates, resulting in biodiversity loss and ecosystem degradation (Lawrence & Wright 2009). The proper functioning of ecosystems depends upon an intricate web of both biotic and abiotic interactions. If this web is degraded by increased human impacts, the processes involved in ensuring a sustainable ecosystem may no longer function effectively (Mooney *et al*. 2009).

Given the limited financial resources available to conserve threatened species and ecosystems, there is a lively debate on how to prioritise species conservation (Crozier 1997; Johnson *et al*. 2005; Wilson *et al*. 2006). Developments in the field of molecular genetics provide us with unprecedented opportunities to explore questions regarding taxonomy, evolution and population genetics of species to better inform conservation and management of threatened species. Determining genetic variability within a species and the geographical distribution of lineages can help in prioritisation of species conservation (Marske *et al*. 2013) (e.g. defining evolutionarily significant units (ESUs) and management units (MSUs) (Moritz 1994)).

Conservation genetics

Conservation genetic research has become an intrinsic component of conservation biology and management of captive and wild populations (Miller & Herbert 2010). Species sociality, population size, dispersal patterns and reproduction affect population persistence and, more specifically, the ability of species to survive natural and anthropogenic change. Traditionally

these ecological traits have been investigated using morphometric, captive or field based studies (Poole & Merchant 1987; Southwell 1987, 1989). Investigating species biology with these traditional techniques has often proven difficult, labour intensive, expensive and time consuming (Southwell 1989; Piggott & Taylor 2003b; Barea-Azcon *et al*. 2007). Molecular techniques are capable of overcoming some of these disadvantages. Furthermore, long-term field studies are often confined to a small part of the species distribution and conclusions based on these single sites may not be directly transferable to other areas of the species distribution (Arnold *et al*. 1993). In these instances molecular techniques may prove beneficial as they are capable of synthesising across multiple spatial scales, from individual to population variation, as well as temporal scales both historical and contemporary (Telfer *et al*. 2006).

The utilisation of genetics in conservation biology can provide unique insights into the ecological and evolutionary processes that have shaped populations and species, as well as providing a means for examining the influences of contemporary climate and environmental change, and predicting the impact of future change (Eldridge 2010). A priority of conservation genetics is to understand and address the impact of the loss of genetic diversity, increased inbreeding and reduced fitness, and consequently a loss of evolutionary potential (Frankham 2005). Determining the distribution and extent of genetic diversity across populations and how it is shaped through time by gene flow, genetic drift, mutation and selection can provide important information for better understanding the effects of recent human impacts (e.g., habitat loss and fragmentation) on species ranges and abundances (Eldridge 2010). As well as assessing a population's "genetic health", molecular genetics techniques can be coupled with ecological, behavioural or geographic data, to create a powerful tool to inform the management of species (Eldridge 2010; Alvarado-Serrano & Knowles 2013).

Molecular techniques assess the level of variation present within the DNA of individuals. The fate of these variations across time and space is influenced by several factors including reproductive success, migration, population size and natural selection, as well as historical and stochastic events (Sunnucks 2000). A suite of molecular markers and techniques are now available depending on the questions being addressed, analytical sensitivity required and the geographic or evolutionary scale under investigation. The recent development and rapid use of next generation sequencing technologies is providing unparalleled access to genomic information (Shendure & Ji 2008). Microsatellite loci can provide information at fine temporal and geographic scales due to their high mutation rate and polymorphism. Conversely, mitochondrial DNA (mtDNA) sequences have a slower mutation rate, making them more suited to examining historical events and relationships (Wan *et al*. 2004). The application of hypervariable microsatellite markers to investigate species ecology has substantially improved our understanding of population biology, behaviour and reproduction in many species, as well as informing conservation initiatives and management plans for many threatened taxa (Eldridge 2010).

Conservation genetics can be applied at three distinguishable levels, that of the species, population and the individual (Sunnucks 2000; Schwartz *et al*. 2007). At the species level, genetics enables the clarification and identification of taxonomic uncertainties, which is fundamental to conserving species (Frankham 1995). Phylogeography has also become an increasingly valuable technique for the identification of species origins, patterns of geographic dispersal and subsequent colonisation(s) (Avise 2000). Determining genetic relationships at a population level is essential for obtaining information on the genetic distinctness of populations, identifying distinct or cryptic lineages, identification of separate management units, populations of high conservation priority and identifying species mating systems

(Frankham *et al*. 2002). Additionally the monitoring of population genetic parameters can provide insights into demographic and evolutionary processes, such as expansions and vicariance events, in natural and captive populations that are difficult or impossible to obtain via traditional methods (Sunnucks 2000; Rubidge *et al*. 2012). Genetic analysis of individuals examines individual fitness traits, identifying source populations for individuals, paternity assignment, reproductive success, and mate choice (Sunnucks 2000). This information can be used collectively to identify appropriate genetically inferred units and scales for management and monitoring (Moritz 2002). Furthermore genetic monitoring of individuals and species offer some of the best opportunities to track populations over time and to evaluate when populations reach critical thresholds that demand management action (Schwartz *et al*. 2007).

Genetics studies of Macropods

The family Macropodidae comprise approximately 63 extant species, which includes kangaroos, wallaroos, wallabies, forest wallabies, hare-wallabies, nail-tail wallabies, rock-wallabies, pademelons, quokka, tree-kangaroos and swamp wallaby (IUCN 2012). The family can be combined with Potoroidae (potoroo, bettong and debatably rat-kangaroos) in the superfamily Macropodoidea, which includes over 70 species (Hume *et al*. 1989). Macropodoidea represents one of the most diverse marsupial assemblages (Dawson 1995). Macropodids range in size from the small hare-wallabies weighing about 1 kg, to the red kangaroo, where males stand at 2 m and weigh more than 80 kg (Hume *et al*. 1989). The habitats into which macropodid species have evolved also cover a broad spectrum from arid central plains and ranges to dense temperate and tropical coastal forests (Hume *et al*. 1989). Macropodids can be naturally found in Australia, New Guinea and some adjacent islands; and

persist outside their native range in New Zealand, Hawaii, Scotland, Germany and England (Hume *et al*. 1989).

The unique biology and iconic status of Macropodoidea has made them of continual scientific interest and obvious candidates for ongoing research (Eldridge *et al*. 2010). Surprisingly, relatively few studies of macropods have used genetic approaches to study their ecology, biogeography and conservation status. Approximately 20 macropodoid species have been the focus of genetic assessment, although most studies have focused on threatened species or those with restricted distributions, such as rock-wallabies (*Petrogale* species) (Eldridge *et al*. 2010). The ecological and biological traits investigated in rock wallabies and other small macropods using genetic studies are shown in Table 1.

In the genus *Macropus* the species that have been genetically studied include the tammar wallaby (*M. eugenii*), red-necked wallaby (*M. rufogriseus*), and the parma wallaby (*M. parma*) (Table 2). To date, the only species of large vagile *Macropus* to have their genetic structure studied are the grey kangaroos (west - *M. fuliginosus* and east - *M. giganteus*) and the red kangaroo (*M. rufus*) (Table 2). As such, many aspects of *Macropus* biology and demography have not yet been investigated using genetic approaches.

Table I-1. Population genetic studies of Macropodoid species (excluding *Macropus*).

Species	**Genetic aspects investigated**	**References**
Rock wallabies		
Petrogale spp.	Taxonomy Species boundaries Hybridisation	(Eldridge & Close 1992; Bee & Close 1993; Spencer & Marsh 1997; Potter *et al*. 2012a)
Allied rock-wallaby (*Petrogale assimilis*)	Genetic diversity Mating systems Sociality and relatedness	(Spencer *et al*. 1995; Spencer *et al*. 1997; Spencer & Marsh 1997; Spencer *et al*. 1998)
Black-footed rock-wallaby (*Petrogale lateralis*)	Genetic diversity Dispersal	(Eldridge *et al*. 1999; Eldridge *et al*. 2001b; Eldridge *et al*. 2004a)
Brush-tailed rock-wallaby (*Petrogale penicillata*)	Population structure Dispersal Population size Mating system Genetic diversity Sociality and relatedness	(Browning *et al*. 2001; Eldridge *et al*. 2001a; Eldridge & Browning 2002; Eldridge *et al*. 2004b; Hazlitt *et al*. 2004; Hazlitt *et al*. 2006; Piggott *et al*. 2006a; Piggott *et al*. 2006b; Hazlitt *et al*. 2010)
Short-eared rock-wallaby (*Petrogale brachyotis*)	Population structure Dispersal	(Potter *et al*. 2012b; Potter *et al*. 2012c)
Yellow-footed rock-wallaby (*Petrogale xanthopus*)	Population structure Sub-speciation	(Pope *et al*. 1996; Eldridge 1997; Zenger *et al*. 2003b)
Other small macropods		
Bettongs Rufous bettong (*Aepyprymnus rufescens*) Northern bettong (*Bettongia tropica*)	Population structure Genetic diversity Dispersal Mating systems Phylogeography	(Pope *et al*. 2000; Pope *et al*. 2005; Pope *et al*. 2012)
Pademelons *Thylogale* spp. Red-necked (*Thylogale thetis*) Red-legged (*Thylogale stigmatica*) Tasmanian pademelon (*Thylogale billardierii*)	Taxonomy Genetic diversity Population structure Hybridisation Phylogeography	(Macqueen *et al*. 2009; Macqueen *et al*. 2010; Eldridge *et al*. 2011; Macqueen *et al*. 2011; Macqueen *et al*. 2012)
Potoroos Gilbert's Potoroo (*Potorous gilbertii*) Long-nosed potoroo (*Potorous tridactylus*)	Genetic diversity Population size Mating system	(Sinclair *et al*. 2002; Frankham *et al*. 2012)
Quokka (*Setonix brachyurus*)	Genetic diversity Population structure	(Sinclair 2001; Alacs *et al*. 2003; Alacs *et al*. 2011)
Rufous hare-wallaby (Lagorchestes hirsutus)	Genetic diversity	(Eldridge *et al*. 2004a)
Tree-kangaroos Lumholtz's tree-kangaroo (*Dendrolagus lumholtzi*) Matschie's tree kangaroo (*Dendrolagus matschiei*) Goodfellow's tree kangaroo (*Dendrolagus goodfellowi*)	Genetic diversity Population structure Taxonomy	(Bowyer *et al*. 2002; McGreevy *et al*. 2009; McGreevy *et al*. 2011, 2012)
Wallabies Brindled nail-tail wallaby (*Onychogalea fraenata*) Swamp wallaby (*Wallabia bicolor*)	Genetic diversity Dispersal Population structure	(Sigg 2006; Paplinska *et al*. 2009)

Table I-2. Population genetic studies of *Macropus* species.

***Macropus* Species**	**Genetic aspects investigated**	**References**
Tammar wallaby (*M. eugenii*)	Genetic diversity Population structure	(Taylor & Cooper 1999; Taylor *et al.* 1999; Eldridge *et al*. 2004a; Miller *et al*. 2011)
Bennett's wallaby (*M. rufogriseus rufogriseus*)	Genetic diversity Population structure	(Le Page *et al*. 2000)
Parma wallaby (*M. parma*)	Genetic diversity	(Taylor *et al*. 1999)
Eastern grey kangaroo (*M. giganteus*)	Genetic Diversity Population genetics Dispersal Hybridisation Phylogeography	(Zenger *et al*. 2003a; Neaves *et al*. 2010; Neaves *et al*. 2013)
Western grey kangaroo (*M. fuliginosus*)	Genetic Diversity Population genetics Dispersal Hybridisation Phylogeography	(Neaves 2007; Neaves *et al*. 2009; Neaves *et al*. 2010; Neaves *et al*. 2012; Neaves *et al*. 2013)
Red kangaroo (*M. rufus*)	Genetic diversity Population structure	(Clegg *et al*. 1998).

The studies that have been conducted on *Macropus* species have investigated a wide range of questions, from identifying founder populations of introduced colonies in New Zealand (Taylor & Cooper 1999) to determining the Australia-wide population genetic structure for larger macropods (Clegg *et al*. 1998). For example genetics has shown the mainland populations of tammar wallaby have retained substantial levels of genetic diversity despite extensive range contractions and population reductions (Eldridge *et al*. 2004a). Taylor and Cooper (1999) determined that the New Zealand tammar wallabies are descendants of an extinct and distinct Australian population using microsatellite loci. The genetic consequences of isolation on island populations of tammar wallaby has also been investigated (Miller *et al*. 2011), revealing low genetic diversity, high levels of effective inbreeding and increased frequency of morphological abnormalities. Genetic analysis was also used to examine the population bottleneck and genetic variation found in an exotic population of Bennett's wallaby in New Zealand (Le Page *et al*. 2000). Another study investigated the genetic diversity and the

retention of reproductive barriers and ecological differences of introduced tammar and parma wallaby populations on a New Zealand island (Taylor *et al*. 1999). Hybridisation between the two species was also investigated using microsatellite date and revealed no evidence of hybridisation (Taylor *et al*. 1999).

Hybridisation has also been investigated in the native range of large macropods. Some evidence of introgression between western grey and eastern grey kangaroos in their sympatric range was identified (Neaves *et al*. 2010). Neaves *et al*. (2010) concluded that for grey kangaroos, introgression is potentially associated with dramatic reductions in densities resulting from variable environmental conditions and reflects occasional genetic leakage rather than the presence of distinct hybrid zones. The eastern and western grey kangaroo have been extensively studied using mtDNA and microsatellites. Western grey kangaroos exhibit the genetic signature of divergence due to unidentified barriers in south-western Western Australia, while previous identified barriers across southern Australia appear to have had little impact despite evidence of broad-scale range expansion (Neaves *et al*. 2012). The continental genetic structure revealed high levels of genetic diversity with limited population structuring across Australia (Neaves *et al*. 2009). Examination of Y-microsatellite loci in western grey kangaroos has revealed high levels of male dispersal with relatively low levels of differentiation, reflective of low effective population size in males due to skewed sex ratios, a polygynous mating system, and a lack of recombination (Neaves *et al*. 2013).

Intraspecific variation, sex-biased dispersal and phylogeography have also been investigated in the eastern grey kangaroo (Zenger *et al*. 2003a). The eastern grey kangaroo has extensive genetic diversity and appears to have had a relatively large long-term effective population size (Zenger *et al*. 2003a). The Tasmanian eastern grey kangaroos are not well differentiated genetically from the mainland eastern grey kangaroos. Although morphological

differences exist, they probably reflect clinal adaptation to environmental differences and/or stochastic effects associated with small founder populations, rather than speciation (Zenger *et al*. 2003a). Weak genetic structure of populations both on a local (<50 km) and regional scale (50-230 km) has been used to infer high levels of dispersal (Zenger *et al*. 2003a). High genetic diversity and low population structure was also found in red kangaroo (Clegg *et al*. 1998). The lack of phylogeographic structure is likely to be the result of long-distance gene flow among existing populations, where sufficient connections have remained over the long term to prevent broad scale phylogeographic structuring (Clegg *et al*. 1998). Genetic structuring is greatest in the relatively wet and topographically complex regions and least in the arid and flat landscapes that dominate much of the continent (Clegg *et al*. 1998). These and future conservation genetic studies will have important implications for the management and conservation of macropod species, particularly in addressing the scale at which management and monitoring should occur (Moritz 1994; Marske *et al*. 2013). Population and conservation genetic studies of macropods are important because of the ~50 macropodoid species, 30 have suffered declines in range, six species are extinct and an additional four have become restricted to offshore islands since European settlement (Calaby & Grigg 1989).

Non-invasive genetic sampling

The application of molecular genetic data provides a powerful tool to elucidate several important aspects of species biology and ecology, including estimates of population size and genetic diversity, the determination of population structure and dispersal patterns and the examination of parentage, relatedness and mating systems (Piggott & Taylor 2003a, b). Molecular approaches have also expanded the methods available for data collection, with sample collection no longer requiring the capture of animals (Taberlet *et al*. 1999; Piggott &

Taylor 2003a, b) and therefore avoiding the negative associations of excessive handling and techniques that stress and alter species behaviour, such as trapping (Taberlet *et al*. 1999; Mathies *et al*. 2001). Of the genetic studies conducted on macropods, only a minority have utilised non-invasively collected samples (Alacs *et al*. 2003; Piggott *et al*. 2006a; Piggott *et al*. 2006b; McGreevy *et al*. 2009; Potter *et al*. 2012b).

The utilisation of DNA from non-invasively collected samples has been used increasingly over the past decade to provide information on population structure, demography, life history, population subdivision, reproduction, sex ratio, diet and disease of rare and elusive animals (Foran *et al*. 1997; Kohn & Wayne 1997; Taberlet *et al*. 1999; Banks *et al*. 2003; Waits & Paetkau 2005; Deagle *et al*. 2010). The primary advantage of utilising non-invasive sampling to obtain DNA is that it allows genetic studies of free-ranging animals without even observing or capturing the species (Taberlet *et al*. 1999). Additional benefits of non-invasive genetic samples over direct sampling include, elevated sample sizes, allowance of natural space-use, unbiased sampling, and reduction of animal stress and capture bias (Sunnucks 2000; Telfer *et al*. 2006; Walker *et al*. 2006).

DNA has been successfully extracted from several non-invasive sources including hair (Constable *et al*. 2001), shed skin (Fetzner 1999), shed feathers (Rudnick *et al*. 2005), faeces (Lucchini *et al*. 2002), urine (Hausknecht *et al*. 2007), egg shell (Martin-Galvez *et al*. 2011), blood spots left on snow (Scandura 2005) and mammal bones from regurgitated owl pellets (Taberlet & Fumagalli 1996). However, one downside of non-invasive genetic samples is that degraded DNA may be encountered, leading to possible difficulties such as genotyping errors and allelic dropout (Taberlet *et al*. 1999). Genotyping errors, if not adequately controlled, can impede accurate estimates of many population metrics (Sunnucks 2000). However, these errors can be overcome with a number of techniques, for example multiple PCR amplifications

to create replication of results (Piggott & Taylor 2003a; Allentoft *et al*. 2011). Therefore, although non-invasive methods are constantly improving (Piggott *et al*. 2004), the time and cost of laboratory analysis can be quite high due to the repeated analysis often being required for degraded samples (Eldridge *et al*. 2010).

Non-invasive methods for the detection of macropods (not necessarily for genetic analysis) have included monitoring hair tubes, arches and snares to determine species presence points (Alacs *et al*. 2003), but have predominately focussed on scats (Coulson & Raines 1985; Johnson & Jarman 1987; Telfer *et al*. 2006, 2008; Styger *et al*. 2011; Wiggins & Bowman 2011). Scats are often the most common and easily collected samples, and are one of the best non-invasive methods for monitoring populations including cryptic and increasingly rare species (Foran *et al*. 1997; Walker *et al*. 2009). An additional benefit of scat samples is that they can be used to identify the food items and parasites in the sample using DNA, enabling analysis of the animal's diet and diseases (Jarman & Wilson 2004; Deagle *et al*. 2010; Ho *et al*. 2010; Wood *et al*. 2013).

There are several critical assumptions that underpin the use of scats in surveys and further genetic analysis, the most important being that the scats of the species must be distinguishable from other sympatric species (Bulinski & McArthur 2000; Telfer *et al*. 2006). For morphologically similar scats, DNA methods are critical for species identification and target the defecator's intestinal cells incorporated into faeces (Cossios & Angers 2006). Several genetic species identification techniques have been developed for non-invasive sampling (Piggott & Taylor 2003a; Bidlack *et al*. 2007). These methods have included the amplification and direct sequencing of mitochondrial DNA (mtDNA) and nuclear DNA using species specific primers and fragment length (Alacs *et al*. 2003; Tobe & Linacre 2008; Oliveira *et al*. 2010), species-specific allele lengths of a given microsatellite (Poetsch *et al*.

2001), or polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) methods (Paxinos *et al*. 1997).

Identification of species or even individuals via DNA extracted from non-invasively collected samples is a potentially important method for obtaining vital information on genetic variation. Non-invasive genetic sampling has been particularly important for conserving small populations of endangered species (Banks *et al*. 2003; Smith *et al*. 2009), but is now also being used for monitoring changes in occupancy and geographical range for more widely dispersed species (McKelvey *et al*. 2006; Ebert *et al*. 2009; Arandjelovic *et al*. 2011). We expect that the use of non-invasive sample collection will soon be used more widely for large-scale surveys that monitor changes in occupancy and geographical range.

The antilopine wallaroo: a large macropodid

Large macropodids comprise the species of herbivorous marsupials weighing between 20 and 90 kg, better known as the "kangaroos" (Dawson 1995). This group contains six species: the eastern grey kangaroo (*Macropus giganteus*), western grey kangaroo (*M. fuliginosus*), red kangaroo (*M. rufus*), common wallaroo (*M. robustus*), black wallaroo (*M. bernardus*) and antilopine wallaroo (*M. antilopinus*). Large macropodids are economically important for ecotourism and the commercial meat trade, but negatively affect agriculture through grazing competition with sheep and cattle (Ho *et al*. 2010). Many species are an important traditional food source for indigenous people as well as holding a strong cultural significance (Murphy & Bowman 2007; Ritchie & Bolitho 2008). With the exception of the black wallaroo, the large macropodids all have extensive geographic ranges across Australia (Ritchie & Bolitho 2008). Although widely distributed, very little is known about the population genetics of many of these iconic species (exceptions are the grey and red kangaroos see above).

The antilopine wallaroo is restricted entirely to the tropical savannahs of northern Australia and occurs in a broad range of vegetation types, ranging from scattered to dense eucalypt woodlands through to tall open forests, all with grass-dominated understoreys (Ritchie 2007). The species' geographical range extends from the Cape York and Einasleigh Uplands bioregions of Queensland, across the top end of the Northern Territory and into the Kimberley region of Western Australia (Ritchie 2007). However, they have a patchy distribution across this range (Ritchie 2007). The antilopine wallaroo is sympatric in parts of its range with both the common and black wallaroos, and the eastern grey and red kangaroos (Ritchie 2008). Although the antilopine wallaroo is highly gregarious (Ritchie 2008) it is often difficult to detect and accurately distinguish from these other large sympatric macropods.

Antilopine wallaroos are longer-limbed and more slender than other wallaroo species, and resemble the grey and red kangaroos in general appearance and behaviour (Ritchie 2008). Males are reddish-tan above and their underside is creamy white; while the tips of the hands and hind feet are black. Females are usually pale grey around the head and forequarters, and have distinctive white fringing of ears. Both sexes have a characteristic large, 'swollen', dark nose, particularly prominent in large males (Ritchie 2007). The availability of permanent water, frequency of fire and the density of eastern grey kangaroos (most likely due to competition for resources) have all been identified as factors influencing the abundance of antilopine wallaroos (Ritchie 2007).

The antilopine wallaroo has been extensively studied across the northern savannahs including; species' socioecology, behaviour, breeding, age structure and habitat use (Russell & Richards 1971; Croft 1987; Poole & Merchant 1987; Ritchie & Bolitho 2008; Ritchie *et al*. 2008; Ritchie *et al*. 2009). However, there is a lack of knowledge regarding the genetic variability, effective population size, population structure and gene flow in the species.

Knowledge of genetic variation and gene flow in antilopine wallaroos is important to understand the processes that have shaped diversity within the Australian tropical savannahs but also to understand the impacts of ongoing habitat depredation, loss and fragmentation due to emerging threats such as changing fire regimes and the rapid expansion of pastoralism and livestock production (Williams *et al*. 2005). Anecdotal evidence suggests that some antilopine wallaroo populations are declining in parts of the Northern Territory and Kimberley region of Western Australia, possibly due to inappropriate fire regimes (Ritchie 2008).

Climate change is predicted to have significant effects on species globally (Walther *et al*. 2002; Hughes 2003), and the antilopine wallaroo has been identified as a species at particular risk due to its restricted range within the tropics (Busby 1988; Ritchie & Bolitho 2008). This species also exhibits highly seasonal reproduction, strongly linked to rainfall in the monsoon season, which may further increase its vulnerability (Ritchie 2007). Ritchie and Bolitho (2008) investigated the effect of climate change on antilopine wallaroos and found that an increase of 2.0°C would result in a reduction of the antilopine wallaroos distribution by 89% with no suitable climactic conditions in Western Australia or the Northern Territory, while a 6.0°C increase results in no suitable climatic conditions in Australia and the possible extinction of the antilopine wallaroo.

Genetic studies can be valuable in identifying the impact of anthropogenic processes and so enable better informed conservation management decisions. Therefore, even though the extinction threat to antilopine wallaroo is presently of minimal conservation concern (IUCN 2012), it is likely to become rare and endangered as a consequence of climate change and changing land-use (Busby 1988; Ritchie & Bolitho 2008). The population genetics of the species has, nonetheless, never been intensely investigated (Ritchie 2007) and limited information is available to guide conservation. Therefore, it is of vital importance that we gain

a stronger understanding of the genetic structure and historical demographic trends of one of Australia's iconic native large tropical herbivores, the antilopine wallaroo, to ensure that any further climate change or environmental change occurs without negatively affecting the persistence of the species (Ritchie *et al*. 2008).

Study aims

The project has five specific aims:

- Develop a quick and inexpensive method to identify macropod scat samples to species level.
- Investigate whether molecular surveys, using non-invasive genetic methods, can be used to obtain occurrence data for fitting ecological models.
- Isolate microsatellite loci for the antilopine wallaroo and design multiplex PCR reactions to enable cost-effective population genetic analyses.
- Assess fine-scale population structure and dispersal of the antilopine wallaroo in Cape York using microsatellite data from field collected scats.
- Determine the phylogeography of antilopine wallaroo by analysing historical aspects of the contemporary spatial distributions of gene lineages in relation to known geographical barriers.

Thesis outline

This thesis utilises non-invasively collected samples to examine the population genetics of one of Australia's large macropods, the antilopine wallaroo, *Macropus antilopinus*. The antilopine wallaroo is Australia's only strictly tropical large macropod and as such may be at greater risk from global change than many other macropods. The research involved utilising non-invasive scat samples to determine the distribution and evolutionary history of antilopine wallaroo. The first step involved accurately identifying a scat sample to species level (Chapter II). Once accurate species identifications of scats were made, the benefits of using molecular surveying of species presence points were investigated (Chapter III). The scats also provided an excellent source of genetic material for further genetic analysis including determining the patterns of dispersal and contemporary population structure (Chapter V). The isolation of microsatellite and the design of multiplex reactions are also critical for genetic monitoring (Chapter IV). To understand the broader phylogeography of antilopine wallaroo across northern Australia, museum samples were also utilised to determine the historical aspects of the contemporary spatial distributions of gene lineages (Chapter VI).

Chapter II presents a new identification test, for macropods, to identify the species from a scat sample. The test utilises a short fragment of the mitochondrial ND2 region that is variable in the species chosen. Carefully selected restriction endonucleases are then applied to cleave the fragment producing a discriminatory fragment pattern size for each species. The test is then trialled on a number of unknown macropod scats to determine species. These species identification points are then compared to the current distribution of each species. This chapter has been published in the journal *Wildlife Research*, and led to a number of radio interviews and a follow-up article in the University of Adelaide's e-science magazine (Appendix II).

Chapter III utilises the molecular identification technique outlined in Chapter II as an indirect genetic survey and assesses its application as an alternative to visual surveys to determine species occurrence. We used generalised linear mixed-effects models to identify ecological correlates of occurrence for four macropod species, across a region of tropical northern Australia, using a non-invasive genetic scat approach with and without additional observation records from visual surveys. The most informative ecological predictors for each species are discussed as well as the reliability and accuracy of the indirect genetic method. This chapter is presented as a completed manuscript and has been submitted to *Austral Ecology*.

Chapter IV outlines the process involved in determining appropriate microsatellite markers for further genetic analysis of scats samples. This includes microsatellite isolation, testing and design of multiplex reactions to increase efficiency for sample processing. This chapter has been published in the journal *Australian Journal of Zoology*.

Chapter V examines the population dynamics of *M. antilopinus* from north-eastern Queensland through the application of high resolution autosomal microsatellite markers. Analysis of genetic information provides an opportunity to address questions regarding demic structure and dispersal to inform on-ground conservation and management decisions. The chapter aims to investigate the genetic diversity in two populations of *M. antilopinus* at the northern and southern end of the species' range in north-eastern Queensland. This chapter is presented as a completed manuscript and will be submitted to *Ecology and Evolution*.

Chapter VI investigates the phylogeography of *M. antilopinus* across its entire distribution of tropical northern Australia by analysing three mitochondrial DNA regions. Divergence time estimates and the effects of known geographic barriers to dispersal are also examined. This

chapter is presented as a completed manuscript and will be submitted to the *Journal of Zoology*.

Chapter VII reviews the significance of the findings from each of these chapters and how they combine to produce a detailed account of the contemporary structure and population history of *M. antilopinus*. The implications of this research to the utilisation of non-invasively collected genetic samples and the management of macropods more generally are also discussed.

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Chapter II: Rapid species identification of eight sympatric northern Australian macropods from faecal-pellet DNA

Macropod scats *(Source: "Tracks, Scats and Other Traces" Trigg 2004)*

Statement of authorship

Rapid species identification of eight sympatric northern Australian macropods from faecal-pellet DNA

Published in *Wildlife Research* May 2013

JESSICA WADLEY (Candidate)

Designed the RFLP identification test, performed DNA extractions, PCR amplifications, RFLP identification tests, sequencing and downstream data processing on all samples, interpreted the data, created figures and tables, and wrote the paper.

I hereby certify that the statement of contribution is accurate

Signed…… Date…..18/12/2013…..

JEREMY AUSTIN

Helped design study, gave advice on laboratory work and edited manuscript

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DAMIEN FORDHAM

Helped design study and edited manuscript

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Wadley, J.J., Austin, J.J. & Fordham, D.A. (2013) Rapid species identification of eight sympatric northern Australian macropods from faecal-pellet DNA.
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Chapter III: Genetic inference as a method for modelling occurrence: A viable alternative to visual surveys

Red kangaroo ©Jessica Wadley

Statement of authorship

Genetic inference as a method for modelling occurrence: A viable alternative to visual surveys

Text in manuscript submitted to *Austral Ecology*

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Performed DNA extractions, PCR amplifications, RFLP identification tests, sequencing and downstream data processing on all samples, ran models, interpreted the results, conducted the cost analysis, created figures and tables, and wrote the paper.

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Helped design study, gave advice on laboratory work and edited manuscript

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Helped design study, determined environmental predictors, created model scripts, interpreted the results and edited manuscript

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Genetic inference as a method for modelling occurrence: A viable alternative to visual surveys

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Abstract

Management and conservation requires a comprehensive understanding of species distributions and habitat requirements. Reliable species occurrence data is critical in the face of climate change and other anthropogenic activity, but is often difficult to obtain or absent, particularly for wide ranging species. This directly affects ecological models of occurrence and habitat suitability and, in turn, conservation and management decisions. We used generalised linear mixed-effects models to identify ecological correlates of occurrence for four macropod species, across a region of tropical northern Australia, using a non-invasive genetic scat approach with and without additional observation records from visual surveys. We show that genetically derived occurrence data, alone, can be used to develop informative ecological models that describe the inter-specific habitat requirements of macropods. Furthermore, we show that genetic scat surveys of macropods are cheaper and less time consuming to conduct,

and tend to provide more occurrence records (and less false absences), then visual surveys. We conclude that indirect surveys using molecular approaches have an important role to play in modelling species occurrence, and developing future management practices and guidelines to aid species conservation.

Keywords

Kangaroo range dynamics; non-invasive genetic sampling; scat; species distribution model; species occurrence.

Introduction

Why are some species present in some locations but not others? This is a fundamental ecological question (Krebs 1972), yet surprisingly, our answers on this point remain far from complete (Parmesan *et al.* 2005; Sagarin and Gaines 2002). Detailed information on factors affecting species' distributions, particularly for wide ranging species, is often absent (Sagarin *et al.* 2006). One reason for the paucity of information on species-habitat relationships is that occurrence data can be costly and time consuming to collect and subject to biases. These data collection issues are potentially amplified when capturing species occurrence responses to regional and temporal variation in environmental conditions (Ritchie *et al.* 2008).

Direct visual survey methods are the most common technique used to explore environmental correlates of species' occurrence. Aerial visual surveys are able to cover large areas, but are often only effective in certain habitat types where visibility is high and for conspicuous species (Cairns *et al.* 2008; Caughley and Grigg 1981; Lundie-Jenkins *et al.* 1999; Southwell and Sheppard 2000). Therefore, many habitats and species are not amenable

to aerial surveys (Southwell *et al.* 1997). Ground surveys, such as walked line transects are able to overcome the terrain and habitat issues of aerial surveys, but are time consuming (Coulson and Raines 1985; le Mar *et al.* 2001; Southwell *et al.* 1995). Driven line transects increase the number of transects able to be monitored and therefore survey coverage (Coulson and Raines 1985; Ritchie *et al.* 2008).

The collection of visual occurrence data from transects relies on two underlying assumptions: (1) objects directly on the transect line are never missed; (2) objects are fixed at their initial sighting position (Southwell *et al.* 1997). However, detectability is influenced by many factors such as sighting conditions and the surveyor's experience (Ringvall *et al.* 2000), potential mismatches between survey time and species diurnal movement, weather patterns, species crypsis and seasonal behaviour (Bonesi and Macdonald 2004). To overcome these issues, statistical adjustments have been developed to account for biases in detection probability when analysing transect surveys (Conroy and Carroll 2009), and inferring patterns and dynamics of species occurrence more generally (MacKenzie *et al.* 2006). Despite these advances, occurrence data from visual transect surveys continues to be used to map species distributions and to identify key environmental and climatic correlates of occurrence without considering biases in detection probability (Gregory *et al.* 2012; Ritchie *et al.* 2008). The problem partly reflects increased access to user-friendly ecological niche modelling software (Elith *et al.* 2011; Thuiller *et al.* 2009), whereby probability of occurrence can be modelled with little thought to underlying uncertainties (Fordham *et al.* 2013).

Direct survey techniques include live-trapping, camera-trapping, scent stations (Barea-Azcon *et al.* 2007; Southwell 1989), as well as visual surveys, and tend to be time consuming and costly. This is especially true for highly vagile species with large distributions (Barea-Azcon *et al.* 2007). Direct surveys can also cause negative physiological effects by disturbing

behavioural and movement patterns of animals (Bleich *et al.* 1994; Mathies *et al.* 2001; Schauster *et al.* 2002). To counter these problems indirect survey techniques are increasingly being used to obtain occurrence data. Indirect monitoring includes the surveying of signs of presence, such as nest or den sites, dig sites, track counts, scat counts or hair counts, to infer presence (Alacs *et al.* 2003; Garcia and Mateos 2009; Kouakou *et al.* 2009). In contrast to visual surveys, indirect techniques are far less labour intensive and can overcome some of the potential sources of bias, such as surveyors experience and species crypsis, associated with traditional direct methods of data collection (Telfer *et al.* 2006). Indirect survey methods for large, terrestrial vertebrates have predominately focused on the presence of scats (Coulson and Raines 1985; Johnson and Jarman 1987; Styger *et al.* 2011; Telfer *et al.* 2006; 2008; Wiggins and Bowman 2011).

Scats are one of the best indirect methods for surveying populations, as they are readily available and easily collected (Foran *et al.* 1997). There are several critical assumptions that underpin the use of scats in surveys, the most important being that scats of the target species must be distinguishable from other sympatric species (Bulinski and McArthur 2000; Telfer *et al.* 2006). For morphologically similar scats DNA methods that target intestinal cells of the animals incorporated into faeces can rapidly provide unambiguous species identification (Cossios and Angers 2006). Several genetic species identification techniques have been developed for non-invasive sampling (Bidlack *et al.* 2007; Piggott and Taylor 2003). These methods have included the amplification and direct sequencing of mitochondrial DNA (mtDNA) (Alacs *et al.* 2003), the use of species-specific mtDNA primers (Tobe and Linacre 2008), species-specific lengths of nuclear fragments (Oliveira *et al.* 2010), species-specific allele lengths of a given microsatellite (Poetsch *et al.* 2001), or polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) methods (Paxinos *et al.* 1997).

Many studies have compared the cost of a variety of direct and indirect survey methods. For example (i) Barea-Azcon *et al.* (2007) compared live trapping, camera-trapping, scent stations and scat detection; (ii) Bonesi and Macdonald (2004) compared direct trapping and indirect survey of signs (scat and tracks) to determine relative abundance; (iii) Schauster *et al.* (2002) looked at the cost of trapping compared to spotlighting, scat deposition and scent stations; (iv) Solberg *et al.* (2006) compared a visual survey technique using a helicopter and a non-invasive genetic method using field collected scats and (v) Carr *et al.* (2012) compared the cost of an infrared survey using a fixed wing aircraft with walking transects and molecular faecal surveys. However, to our knowledge, no study has evaluated the ability of genetic methods to provide reliable presence data as well as information on the factors affecting species' occurrence patterns from a cost benefit perspective.

As part of a broader macroecological study, we used two different methods to survey four large, vagile, sympatric macropods, *Macropus antilopinus* (antilopine wallaroo), *M. agilis* (agile wallaby), *M. robustus* (common wallaroo), and *M. giganteus* (eastern grey kangaroo) across northern Queensland, Australia. We examine the suitability of using occurrence data from an indirect survey (non-invasive genetic identification of scats) to determine species occurrence-habitat relationships with and without additional occurrence data from a direct field visualisation survey (driving transects). Our specific objectives were: (1) to conduct both visual and indirect genetic occurrence surveys of large macropods across north-eastern Australia; (2) explore whether indirect genetic occurrence data can be used to fit ecological models of occurrence for macropods; (3) use these models to establish primary environmental and climatic correlates of occurrence for each macropod species in our study region; and (4) compare the cost of visual versus indirect genetic surveys.

Methods

We undertook 51 transect surveys of macropods in Queensland, Australia (Fig. 1). Transects were spread across four Köppen climate zones; equatorial (number of transects (n) = 19), tropical (n = 17), subtropical (n = 9), and grassland (n = 6). The macropod species of interest were *Macropus agilis*, *M. antilopinus*, *M. giganteus* and *M. robustus*. Survey site locations were chosen primarily to establish range determinants of *M. antilopinus*, however they provide a good representation of environmental and climatic conditions affecting macropods more generally across north-eastern Australia (Menkhorst and Knight 2010).

Figure III-1. Map of north-eastern Australia showing transect sites used for macropod surveys. Black diamonds represent the 51 transect locations. Climatic layers based on the Köppen classification are also shown; brown = equatorial, dark green = tropical, light green = subtropical, orange = grassland. Insert shows the location within Australia.

Visual survey

The visual macropod survey techniques were based on Ritchie *et al.* (2008). Each transect consisted of 5 kms of road, infrequently used by other vehicles. Based on previous macropod occurrence surveys (Ritchie *et al.* 2008), each transect was surveyed four times over two consecutive days in the morning and evening. Morning surveys spanned 0630-0930 and evening surveys spanned 1600-1830 to coincide with the time when macropods are most active (Ritchie *et al.* 2008). Transects were driven at <10 km/h and macropods were recorded on either side of the transect line by two observers in the tray of an open vehicle. To avoid observer bias, the same trained observers were used for all surveys. When a macropod was sighted, the species and GPS location was recorded.

Indirect genetic survey

Four spatially separated scats were collected at every 1 km point along each transect with fresh scats being preferentially collected. All scats collected were georeferenced using hand-held GPS (WGS 84). Scat samples were placed in individual paper bags in the field and stored in plastic boxes with a silica desiccant before being transported to the University of Adelaide and then frozen at -20 °C. A total of 245 scats from the 51 transects were selected for DNA extraction and species identification, one from each available 1 km point. Note that scats were unable to be located at all 1 km intervals. Species identification of scats was conducted following Wadley *et al.* (2013), whereby a small amount (2 cm^2) of the outer surface of each scat was scraped and removed using a sterile scalpel and 'Bioline fecal ISOLATE' kits (Bioline, London, UK) were used for extracting DNA. Extractions were performed in a DNA extraction laboratory physically separate from PCR-setup and post-PCR laboratories to prevent cross contamination with previously amplified DNA and all extractions included a negative control. All DNA extracts and negative controls were amplified for a 275-bp mtDNA

ND2 fragment using macropod specific PCR primers (M496 5'-GCTTGAATAACAAACCAATGAACCC-3'/M497 5'-CCTCCTCAGCCTCCGAGYAT-3'), digested with three restriction endonucleases, (*AluI HphI* and *BstNI*) and examined by gel electrophoresis to determine species (Wadley *et al.* 2013).

Vegetation surveys

Vegetation surveys were conducted on all transects at 1 km intervals using four 1 x 1 m wire quadrants (Ritchie *et al.* 2008). Two quadrants were placed on either side of each transect at a distance of 25 m and 50 m from the road. The four quadrants were then surveyed for percentage grass cover, grass height and biomass. Percentage grass cover was a comparison of vegetated area versus bare ground within the quadrant. Grass height consisted of two measurements; one conducted in the field to produce quadrant grass height and one from the cut sample used in the biomass calculations. To determine biomass, $\frac{1}{8}$ of a 1 x 1 m transect was cut and dried for 24 hours in an oven at 60 °C. Weight was measured to 5 g accuracy using a 1 kg Super Samson, Salter Scale. For each quadrant, biomass was then calculated as

$$Quadrant\ biomass = \left(\left(\frac{qgh}{wsgh}\right) * (sb * 8)\right) * (\frac{gcp}{100})$$

Where 'qgh' is quadrant grass height. 'wsgh' is weighed sample grass height. 'sb' is sample biomass. 'gcp' is grass cover percentage. The average of the quadrant biomasses for each transect (n = 24) was then calculate to give transect biomass.

Environmental predictors

A candidate set of environmental predictors of macropod presence/absence was selected using published literature (Ritchie and Bolitho 2008; Ritchie *et al.* 2009; Ritchie *et al.* 2008) and expert advice. To avoid co-linearity among predictors, variables with a spearman's Rank ≥ 0.7

were excluded from the analysis. Where possible, we favoured retaining variables with the strongest ecological support (see Supplementary Table S1). We considered predictors as two classes of variables (1) *habitat variables*, consisting of slope (in degrees from horizon), distance to closest permanent water (water, in hundred km), relative biomass (biomass), and elevation (elev, metres above sea level); (2) *human variables*, consisting of land use (grazing land or non-grazing land) and fire (number of years the land burnt from 1997-2010, in the late dry season).

Statistical analysis

We used generalised linear mixed-effects models (GLMM) with a binomial distribution to identify ecological correlates of species occurrence using (i) molecular presence/absence records (*molecular identification*) and (ii) a combination of molecular and visual presence/absence records (*combined identification*), separately. The *combined identification* records included any additional presence points from the visual survey that were not detected from the indirect genetic survey. We did not model occurrence using visual-only records because models did not meet linear assumptions (see below) for some species. Likelihood ratio tests for mixed-effects models (Crainiceanu and Ruppert 2004) confirmed that Köppen *climate zone* should be treated as a random effect, not a fixed effect. Thus, models were not simplified to generalised linear models (Pinheiro and Bates 2000). To avoid over-parameterising models, due to the relatively small samples size of occurrence records for each species (n = 51), we initially modelled the likely effect of non-climate habitat variables and anthropogenic human variables separately. After establishing the most parsimonious habitat and anthropogenic human models we then explored, more generally, the relative importance of these types of conditions on occurrence. We have used similar approaches elsewhere (Mellin *et al.* 2012). We compared and ranked models using Akaike's information criterion corrected

for small sample size (AICc) (Burnham and Anderson 2002). We assessed each model's probability relative to the entire model set using AICc weights (wAICc), and its structural goodness-of-fit with per cent deviance explained (DEV). All residual distributions met linear model assumptions (assessed using normalised scores of standardised residual deviance using Q-Q plots). All models were implemented using *lme4* package (Bates *et al*. 2012) in the statistical software package R (R Development Core Team 2012).

Cost analysis

To compare the economic feasibility of using indirect genetic and visual identification methods we examined the retrospective cost of the two methods. We considered observed expenses for field and laboratory work, salaries and car hire. We acknowledge that costs will be somewhat case study specific. For simplicity we excluded variables such as flights to and from field sites and the purchase of field-work equipment as they were common to both techniques. Although the site surveys were the same for both techniques, we estimate that the duration of time needed for indirect genetic surveys would be substantially less than for the visual surveys. This is because the visual surveys require participants to be located at each site, surveying up to two transects at a time, for at least two days. Visual surveys can only take place in the morning and evening when macropods are most active. Depending on the travel distance between sites an extra day at each site is often required to find and set up each transect and camp. For the indirect genetic survey, sites only need to be visited for a minimal amount of time to ensure transects are located and set up. Scats can be collected and habitat surveyed at each interval point regardless of the time of day. This can result in several transects and sites being completed each day (depending on distance between sites) resulting in a significantly shorter field trip duration compared to the visual survey. The cost involved in hiring a four-wheeled drive vehicle for the surveys (visual or indirect genetic) is influenced by

the duration of the fieldwork, costed at a daily rate of $195 (AUD), including fuel and safety equipment. Research assistants are needed for fieldwork, costed at $61,880 pa (AUD). For the visual survey three people are required, one to drive and two observers in the tray of the vehicle (Ritchie *et al.* 2008). However, the indirect genetic method only requires two people for the duration of field work.

The indirect genetic method then requires samples to undergo laboratory analysis for identification of species. For this study samples were processed in-house (Wadley *et al.* 2013), however, for the cost benefit analysis we have used commercial costs from an external source (the Australian Genome Research Facility - AGRF) to provide a comparison of surveying cost for those without access to the required laboratory equipment. The laboratory work costing associated with identifying each sample for in-house analysis is described in detail in Supplementary Table S2.

Results

Species occurrence

Both visual and indirect genetic survey methods each identified independently four macropod species occurring across the surveyed region in north-eastern Australia (Fig. 2). For all species the indirect genetic method provided more presence locations compared to the visual method (i.e., along transects where species were not detected by visual surveys, indirect genetic evidence showed that they do occur in the area). *Macropus antilopinus* was recorded at 21 sites based on the indirect genetic method while the visual survey only identified the species at 15 sites. Indirect genetic methods identifying *M. agilis* at 27 sites, *M. giganteus* at 10 sites and *M. robustus* at 21 sites compared to visual methods with 16, 7 and 18 sites respectively. The visual survey technique only identified two additional presence points (one for *M. giganteus* and one for *M. robustus*) compared to the indirect genetic survey (Fig. 2c, d), with the *M. giganteus* point possibly being the result of misidentification due to its location being well outside its established range.

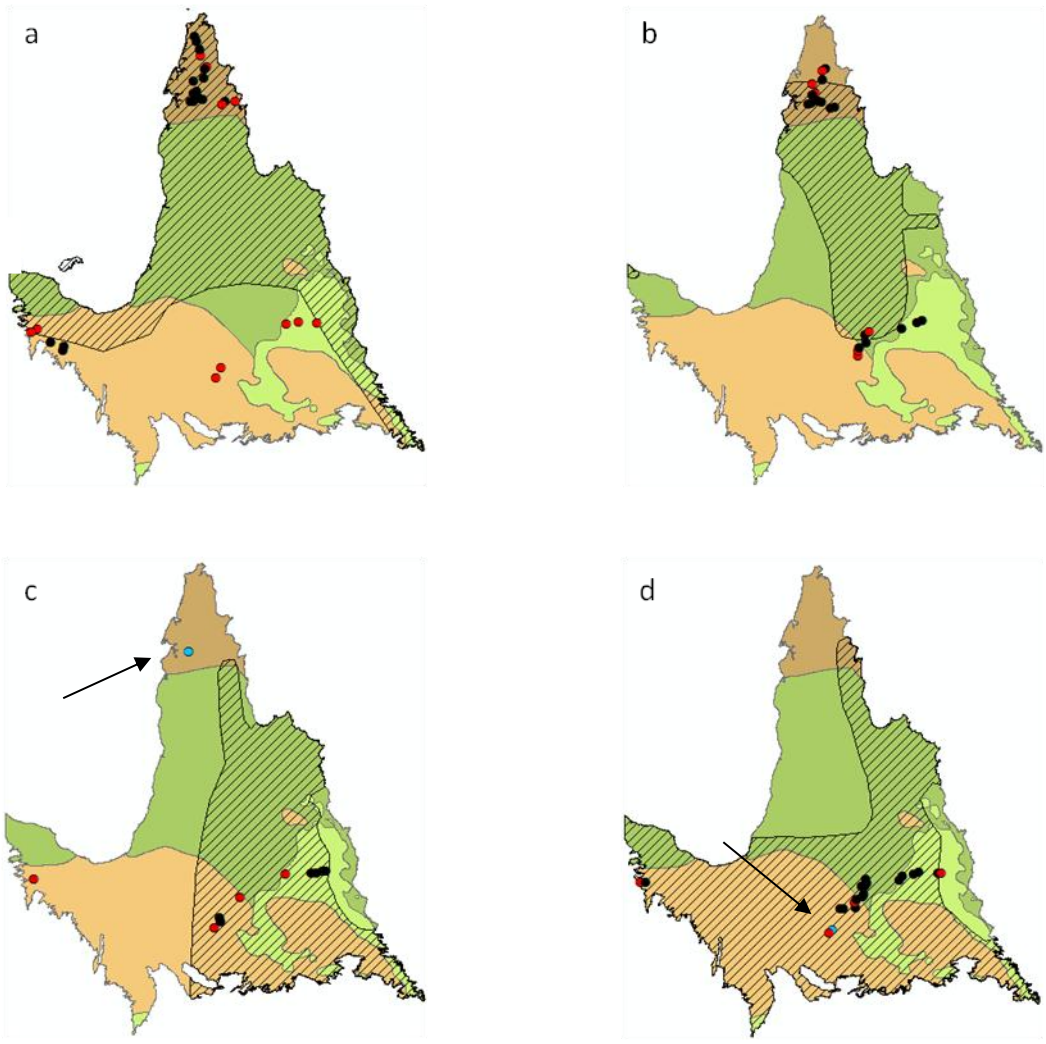

Figure III-2. Map showing the location of species presence points based on indirect genetic and visual surveys. a = *Macropus agilis*, b = *M. antilopinus*, c = *M. giganteus*, d = *M. robustus*. Black dots represent presence points identified by both survey techniques. Red dots represent presence points identified by indirect genetic methods only. Blue dots shown via arrows represent presence points identified by visual methods only. Köppen classifications (coloured background area) are the same as Fig. 1. Each species current distribution based on the IUCN Redlist is shown in black diagonal lines (IUCN 2012).

Occurrence models

Our models show that the *molecular identification* records can be used to explore how environmental conditions influence the occurrence of macropods (Table 1). The most parsimonious occurrence models explained between 17 – 43 % of model structural deviance, depending on the species.

Table III-1. GLMM model results for each species using occurrence data from molecular identification records.

Species	Model type	Model	AICc	K	dAIC	wAIC	DEV
M. antilopinus	human	land use + (1|zone)	66.39	4	0.00	0.60	16.77
		land use + fire + (1|zone)	67.39	5	1.00	0.36	18.88
	habitat	(1|zone)	73.49	3	0.00	0.30	3.08
		biomass + (1|zone)	74.64	4	1.15	0.17	4.86
		slope + (1|zone)	75.23	4	1.74	0.13	3.97
M. agilis	human	land use + (1|zone)	63.60	4	0.00	0.56	22.40
		(1|zone)	65.59	3	1.99	0.21	16.24
		land use + fire + (1|zone)	66.06	5	2.46	0.16	22.40
	habitat	elev + (1|zone)	62.53	4	0.00	0.46	23.91
		elev + biomass + (1|zone)	64.14	5	1.61	0.21	25.16
	human + habitat	elev + land use + (1|zone)	61.19	5	0.00	0.34	29.30
		elev + biomass + land use + (1|zone)	61.75	6	0.56	0.26	32.23
		elev + (1|zone)	62.53	4	1.34	0.18	23.91
M. robustus	human	(1|zone)	52.96	3	0.00	0.52	32.78
		land use + (1|zone)	54.72	4	1.76	0.21	33.65
		fire + (1|zone)	54.95	4	1.99	0.19	33.31
	habitat	elev + (1|zone)	48.26	4	0.00	0.47	43.00
		slope + (1|zone)	49.96	4	1.71	0.20	40.54
		elev + biomass + (1|zone)	50.43	5	2.17	0.16	43.47
M. giganteus	human	(1|zone)	49.99	3	0.00	0.53	13.87
		land use + (1|zone)	51.69	4	1.69	0.23	15.18
		fire + (1|zone)	52.35	4	2.36	0.16	13.87
	habitat	biomass + water + slope + elev + (1|zone)	45.58	7	0.00	0.55	42.72
		slope + (1|zone)	48.11	4	2.53	0.16	22.27

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. Habitat variables (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). Human variables (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). Only models with wAIC > 0.1 are shown.

Anthropogenic human processes are likely to have a larger influence on the occurrence of *M. antilopinus* than habitat variables in our study area. Land use had the most pronounced influence on *M. antilopinus* occurrence (~ land use $^{+}$ [1|zone]: DEV = 17 %). There was also good support for including fire in a multi-term model (~ land use $^{+}$ fire $^{+}$ [1|zone]: dAICc = 1; DEV = 18.88 %).

The most parsimonious model for *M. agilis* occurrence included habitat variables (elevation) as well as anthropogenic human processes (land use) (elev $^{+}$ land use $^{+}$ [1|zone]: DEV = 29.90%). There was also good support for including biomass in the multi-term model (elev $^{+}$ biomass $^{+}$ land use $^{+}$ [1|zone]; $dAIC_c$ = 0.56; DEV = 32%) and for modelling occurrence as a single-term model using elevation (elev $^{+}$ [1|zone]; $dAIC_c$ = 1.34; DEV = 24%), alone.

Anthropogenic human processes did not strongly influence the occurrence of *M. robustus* in our study area. Elevation had the largest influence on *M. robustus* occurrence (elev $^{+}$ [1|zone]; DEV = 43%). There was also good support for using slope as a predictor of *M. robustus* occurrence (slope $^{+}$ [1|zone]; $dAIC_c$ = 1.71; DEV = 41%).

Habitat variables also had the strongest influence on the occurrence of *M. giganteus*. A multi-term model that included biomass, distance to water, slope and elevation explained the greatest variation in occurrence (biomass $^{+}$ water $^{+}$ slope $^{+}$ elev $^{+}$ [1|zone]: DEV = 43%). There was less support for the next ranked model (slope $^{+}$ (1|zone): $dAIC_c$ = 2.53; DEV = 22%). The GLMM results tables for all candidate models are shown in Supplementary Tables S3-S6.

Combining indirect genetic and visual survey observations (i.e., using the *combined identification* records) for *M. robustus* and *M. giganteus* (the two species where visual surveys provided additional presences) resulted in similar conclusions to the *molecular identification*

results (Supplementary Table S7). The most parsimonious model for *M. robustus* was ~ elev $^{+}$ (1|zone) (DEV = 45%); and for *M. giganteus*, it was ~ biomass $^{+}$ water $^{+}$ slope $^{+}$ elev $^{+}$ (1|zone) (DEV = 32%). Interestingly, combining visual and indirect genetic survey data resulted in a poorer model structural fit for *M. giganteus* compared to the *molecular identification* model. This may be due to an inaccurate visual identification record for *M. giganteus* in far northern Australia (Fig. 2c) affecting the fit of the model.

Cost comparison

The cost involved in collecting and identifying the non-invasively collected scat samples for the molecular identification survey was substantially less than the cost involved in conducting a direct visual survey of macropod occurrence (Table 2). The time required to complete the visual survey of the sites was 120 days, while we expect that the field work for the indirect genetic survey would only take 30 days. Even with the added component of out-sourcing laboratory analysis from a commercial company, the indirect genetic surveys would take less time to complete and cost significantly less than the visual surveys (Table 2), while also providing important additional presence data (see above). Therefore, the indirect genetic method would be able to survey over three times as many sites as the visual survey for the same cost and time. For researchers with access to in-house genetic facilities the molecular work is substantially cheaper than outsourcing and would provide additional cost savings (See Supplementary Table S2).

Table III-2. Cost comparison of indirect genetic and visual survey techniques for 51 sites across north-eastern Australia. All prices are in Australian dollars.

Category of cost involved	Visual survey		Indirect genetic survey	
	Description	Total	Description	Total
Field work Salary	x3 people for 120 days $61,880 per annum $20,344 x3	$61,032	x2 people for 30 days $61,880 per annum $5,086 x2	$10,172
Four wheeled drive vehicle hire	Field work 120 days $195 per day	$23,400	Field work 30 days $195 per day	$5,850
Laboratory analysis		N/A	Commercial service provided by AGRF (extraction through to identification) $40 per sample x245 samples	$9,800
Total		$84,432		$25,822

Discussion

Understanding the biological and environmental factors that limit the distribution of organisms requires occurrence or abundance data (Franklin 2009), unless the fundamental niche of a species can be mechanistically linked to key organismal traits using biophysical models (Kearney *et al.* 2008). Indirect genetic surveys are a cost effective method for collecting species occurrence data, which can be directly used in ecological models to evaluate potential drivers of species distributions. The results from these models can then be potentially used to better manage species today and in the future (Araujo and Peterson 2012).

Previous macropod studies have used scat count data to develop ecological models of habitat use (Telfer *et al.* 2006; Wiggins and Bowman 2011), however, they did not utilise molecular methods of identification. Consequently, their results are likely to include model biases and uncertainties brought about by potential problems of misidentifying species with similar scat morphology (Telfer *et al.* 2006). In contrast, these problems are largely avoided by using genetically inferred occurrence data to build ecological models. The genetic species identification test allows the depositor of a scat to be identified accurately (i.e., without bias due to the observer; Ringvall *et al.* 2000). However, if a suspicious identification is determined, the indirect genetic survey has the added benefit of being able to independently double check the sample via further genetic analysis, increasing the reliability attached to that sample. For example, the out-of-range *M. giganteus* scat sample was reanalysed using sequencing and identified as *M. giganteus* again, confirming the accuracy of the species identification: something that could not be done with the out-of-range *M. giganteus* visual observation. The out-of-range *M. giganteus* visual observation is therefore still questionable and should be used with caution.

The overall cost of the indirect genetic survey was considerably less than the visual survey, even with the added cost of the molecular analysis. Both were able to identify all four species of interest. However, the indirect genetic method revealed important additional presence points for all four species. Thus indirect genetic occurrence data is cheaper to collect, is more reliable because the identity of samples can be easily cross-checked and can potentially provide more occurrence points when compared to visual transect methods. In wildlife surveys, whether they are based on scat counts or observations of animals, invariably there is a chance of recording 'false absences' by not detecting the presence of a species in an area that it in fact inhabits (MacKenzie *et al.* 2002). Although statistical techniques have been developed to account for many inherent biases in occupancy data (MacKenzie *et al.* 2006), ecological niche models (also referred to as species distribution models and bioclimatic envelope models) (Peterson *et al.* 2011) continue to be developed with little consideration of uncertainties in presence-only or presence/absence data.

By utilising genetic methods to collect data on species' occurrence, the chance of missing species' at site or misidentifying a species is decreased. The former is particularly important for more widespread, vagile species that can often be missed on transect surveys. Indirect genetic surveys utilising scats have previously been used to determine reliable species identification (Alacs *et al.* 2003; Berry *et al.* 2007), calculating species abundance (Brinkman *et al.* 2011; Mills *et al.* 2000; Poole *et al.* 2011), determine species distributions (Ruiz-Gonzalez *et al.* 2008) and estimating population size (Kohn and Wayne 1997; Piggott *et al.* 2006; Solberg *et al.* 2006). This study has taken the indirect genetic method further by using statistical models to investigate the environmental correlates of genetically inferred occurrence data, showing that molecular scat identification provides a promising source of presence/absence data for constructing ecological niche models.

All four macropod species have distributions across tropical Queensland. However, both *M. giganteus* and *M. robustus* are also found in non-tropical climates. *Macropus giganteus* is found in the temperate regions of eastern Australia, and *M. robustus* has a distribution covering the majority of Australia including the central arid deserts. Our results indicate that for these two species, habitat variables are better predictors of occurrence than human variables, at least in north-eastern Australia. Both elevation and slope influence *M. robustus* occurrence while the added effects of permanent water and relative grass biomass were important for the more temperate species, *M. giganteus*. Our results support previously hypothesised drivers of range dynamics for *M. giganteus*. For example, Dawson *et al.* (2004) concluded that the creation of artificial watering points aid the expansion of *M. giganteus* into more arid habitats; and Caughley *et al.* (1988) suggested that food (relative grass biomass) could determine the inland boundary of *M. giganteus*.

Elevation and land use influence the occurrence of *M. agilis* whose distribution is limited to tropical northern Australia. The human variables of land use and fire regimes were the best predictors of occurrence for *M. antilopinus* which is also confined to the tropical regions of northern Australia. Ritchie *et al.* (2008) also found fire to be a good predictor of *M. antilopinus* presence across northern Australia. By showing the important effect of human variables on the occurrence of two endemic tropical macropods (*M. agilis* and *M. antilopinus*), we provide additional evidence of the potential impact of land conversion and altered fire regimes on mammals in northern Australia (Woinarski *et al.* 2011). More specifically, our results reiterate the potential vulnerability of these species to anthropogenic change (Williams *et al.* 2005). However, to determine the range-wide influence of land use and fire regimes on *M. agilis* and *M. antilopinus* occurrence and vulnerability, data from across the species' ranges would be required.

Surveying the occurrence and abundance of species is critical to the design and monitoring of management and conservation programmes (Clarke *et al.* 2001). Therefore, methods that produce more reliable occurrence data (i.e. occurrence data that does not require pre-analyses to account for inherent biases) using cost-effective techniques, such as indirect genetic surveys, can directly benefit both management and conservation. For example, ecological niche models are the primary tools used to determine species' habitat requirements and availability, and how these may be altered by climate change (Araujo and Peterson 2012; Franklin 2009; Rodríguez-Castañeda *et al.* 2012). Given that the predictive capacity of ecological niche models is influenced by uncertainties in occurrence data (Syfert *et al.* 2013), and that models are often built without considering these biases, we anticipate that genetically inferred occurrence data will strengthen niche model predictions, including previous efforts to model climate change impacts on macropods (Ritchie and Bolitho 2008).

Conclusion

Indirect genetic surveys are able to generate reliable data on species occurrence in a cost effective manner. Not only is the expense of conducting indirect genetic surveys of macropods significantly less than visual driving-transect surveys, genetically derived occurrence records can be used to model species' habitat-occurrence relationships. Our results extend well beyond establishing the primary correlates of occurrence for macropod species in north-eastern Australia. Forecasts of habitat suitability using occurrence data are already being used to inform species conservation, establish protected reserve networks (Araujo *et al.* 2011) and guide the conservation of threatened species (Fordham *et al.* 2012). Therefore indirect surveys using molecular approaches have an important role to play in developing future management practices and guidelines to aid species conservation.

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Supplementary Material

Table S1. Data sources for the predictors used to model occurrence.

Predictor	Unit of measure	Data Source
Slope	Degrees from horizon	Slope was calculated from Geosciences 9 second Digital Elevation Model (DEM) http://www.ga.gov.au/meta/ANZCW0703011541.html
Water	Distance to closest permanent water in hundred km	Water data was taken from Geosciences Australia's 250K topo permanent water data https://www.ga.gov.au/products/servlet/controller?event=GEOCAT_DETAILS&catno=64058
Biomass	Dry weight per area	Biomass was calculated from measurement collected in the field using $$Quadrant\ biomass = \left(\left(\frac{qgh}{wsgh}\right) * (sb * 8)\right) * (\frac{gcp}{100})$$ Where 'qgh' is quadrant grass height. 'wsgh' is weighed sample grass height. 'sb' is sample biomass. 'gcp' is grass cover percentage. The average of the quadrant biomasses for each transect (n = 24) was then calculate to give transect biomass.
Elevation	Metres above sea level	Elevation was taken from Geosciences 9 second Digital Elevation Model (DEM) http://www.ga.gov.au/meta/ANZCW0703011541.html
Land use	Grazing land or non-grazing land	Land use is taken from Australian Bureau of Agriculture and Resource Economics – Bureau of Rural Sciences (ABARE-BRS): Catchment Scale Land Use of Australia – Update March 2010 http://adl.brs.gov.au/anrdl/metadata_files/pa_luausr9abll07611a00.xml
Fire	Number of years the land burnt from 1997-2010 in the late dry season	Fire data taken from Landgate (http://www.landgate.wa.gov.au/), from 1 km resolution NAOO/AVHRR satellite imagery

Table S2. In-house lab work cost for identifying indirect scat samples. Process includes extraction of scat DNA, PCR and RFLP analysis but does not take into account salary or lab set up costs.

Lab procedure	**Item description**	**Cost**	**Unit of measure**	**Cost per sample**
Extraction	Bioline fecal extraction kit	$345	100 reactions	$3.45
	Consumables (plastic wear, tips etc.)			$1.25
PCR	Taq, reagents and consumables			$0.90
PCR gel	1 MD gel	$7.52	Contains 38 samples	$0.20
RFLP	*AluI* endonuclease	$78.30	1000 units	$0.23
	HphI endonuclease	$74.70	1000 units	$0.22
	BstNI endonuclease	$68.40	3000 units	$0.14
RFLP gel	1 MD gel	$7.52	Contains 36 samples	$0.21
Total per sample for identification	Extraction, PCR plus gel and RFLP plus gel			$6.60

Table S3. GLMM results for all *M. antilopinus* candidate models using occurrence data from molecular identification records.

Identification type	Model type	Model	AICc	K	dAIC	wAIC	DEV
Molecular	human	land use + (1|zone)	66.39	4	0.00	0.60	16.77
		land use + fire + (1|zone)	67.39	5	1.00	0.36	18.88
		(1|zone)	73.49	3	7.10	0.02	3.08
		fire + (1|zone)	73.88	4	7.49	0.01	5.93
		null	75.62	2	9.23	0.01	0.00
Molecular	habitat	(1|zone)	73.49	3	0.00	0.30	3.08
		biomass + (1|zone)	74.64	4	1.15	0.17	4.86
		slope + (1|zone)	75.23	4	1.74	0.13	3.97
		null	75.62	2	2.13	0.10	0.00
		elev + (1|zone)	75.78	4	2.29	0.10	3.17
		water + (1|zone)	75.84	4	2.36	0.09	3.09
		elev + biomass + (1|zone)	76.84	5	3.35	0.06	5.25
		biomass + water + (1|zone)	77.11	5	3.62	0.05	4.86
		biomass + water + slope + elev + (1|zone)	81.38	7	7.89	0.01	6.36

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. *Habitat variables* (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). *Human variables* (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). We include the null model (null).

Table S4. GLMM results for all *M. agilis* candidate models using occurrence data from molecular identification records.

Identification type	Model type	Model	AICc	K	dAIC	wAIC	DEV
Molecular	human	land use + (1|zone)	63.60	4	0.00	0.56	22.40
		(1|zone)	65.59	3	1.99	0.21	16.24
		land use + fire + (1|zone)	66.06	5	2.46	0.16	22.40
		fire + (1|zone)	67.87	4	4.28	0.07	16.34
		null	77.04	2	13.44	0.00	0.00
Molecular	habitat	elev + (1|zone)	62.53	4	0.00	0.46	23.91
		elev + biomass + (1|zone)	64.14	5	1.61	0.21	25.16
		biomass + (1|zone)	65.53	4	3.00	0.10	19.68
		(1|zone)	65.59	3	3.06	0.10	16.24
		biomass + water + slope + elev + (1|zone)	67.57	7	5.04	0.04	27.82
		slope + (1|zone)	67.89	4	5.36	0.03	16.31
		biomass + water + (1|zone)	67.92	5	5.39	0.03	19.81
		water + (1|zone)	67.94	4	5.41	0.03	16.25
		null	77.04	2	14.51	0.00	0.00
Molecular	human + habitat	elev + land use + (1|zone)	61.19	5	0.00	0.34	29.30
		elev + biomass + land use + (1|zone)	61.75	6	0.56	0.26	32.23
		elev + (1|zone)	62.53	4	1.34	0.18	23.91
		land use + (1|zone)	63.60	4	2.40	0.10	22.40
		elev + biomass + (1|zone)	64.14	5	2.95	0.08	25.16
		(1|zone)	65.59	3	4.39	0.04	16.24
		null	77.04	2	15.84	0.00	0.00

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. *Habitat variables* (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). *Human variables* (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). We include the null model (null).

Table S5. GLMM results for all *M. robustus* candidate models using occurrence data from molecular identification records.

Identification type	Model type	Model	AICc	K	dAIC	wAIC	DEV
Molecular	human	(1|zone)	52.96	3	0.00	0.52	32.78
		land use + (1|zone)	54.72	4	1.76	0.21	33.65
		fire + (1|zone)	54.95	4	1.99	0.19	33.31
		land use + fire + (1|zone)	56.75	5	3.78	0.08	34.28
		null	75.62	2	22.65	0.00	0.00
Molecular	habitat	elev + (1|zone)	48.26	4	0.00	0.47	43.00
		slope + (1|zone)	49.96	4	1.71	0.20	40.54
		elev + biomass + (1|zone)	50.43	5	2.17	0.16	43.47
		biomass + (1|zone)	52.86	4	4.61	0.05	36.36
		(1|zone)	52.96	3	4.71	0.05	32.78
		water + (1|zone)	53.92	4	5067	0.03	34.80
		biomass + water + slope + elev + (1|zone)	54.27	7	6.02	0.02	45.58
		biomass + water + (1|zone)	54.75	5	6.50	0.02	37.21
		null	75.62	2	27.36	0.00	0.00

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. *Habitat variables* (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). *Human variables* (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). We include the null model (null).

Table S6. GLMM results for all *M. giganteus* candidate models using occurrence data from molecular identification records.

Identification type	Model type	Model	AICc	K	dAIC	wAIC	DEV
Molecular	human	(1|zone)	49.99	3	0.00	0.53	13.87
		land use + (1|zone)	51.69	4	1.69	0.23	15.18
		fire + (1|zone)	52.35	4	2.36	0.16	13.87
		land use + fire + (1|zone)	54.14	5	4.15	0.07	15.20
		null	56.99	2	7.00	0.02	0.00
Molecular	habitat	biomass + water + slope + elev + (1|zone)	45.58	7	0.00	0.55	42.72
		slope + (1|zone)	48.11	4	2.53	0.16	22.27
		elev + biomass + (1|zone)	49.67	5	4.08	0.07	24.13
		(1|zone)	49.99	3	4.41	0.06	13.87
		elev + (1|zone)	50.26	4	4.68	0.05	18.00
		biomass + (1|zone)	50.55	4	4.96	0.05	17.48
		biomass + water + (1|zone)	51.28	5	5.70	0.03	20.92
		water + (1|zone)	51.52	4	5.94	0.03	15.52
		null	56.99	2	11.41	0.00	0.00

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. *Habitat variables* (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). *Human variables* (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). We include the null model (null).

Table S7. GLMM results for all *M. robustus* and *M. giganteus* candidate models using occurrence data from combined identification records.

Species	Model type	Model	AICc	K	dAIC	wAIC	DEV
M. robustus	human	(1|zone)	52.96	3	0.00	0.58	33.40
		land use + (1|zone)	54.70	4	1.75	0.22	34.28
		fire + (1|zone)	55.13	4	2.17	0.18	33.66
	habitat	elev + (1|zone)	46.93	4	0.00	0.57	45.43
		elev + biomass + (1|zone)	48.90	5	1.98	0.21	46.17
M. giganteus	human	land use + (1|zone)	56.38	4	0.00	0.36	10.67
		(1|zone)	56.40	3	0.03	0.35	6.18
		fire + (1|zone)	58.76	4	2.38	0.11	6.19
		land use + fire + (1|zone)	58.80	5	2.42	0.11	10.75
	habitat	biomass + water + slope + elev + (1|zone)	52.90	7	0.00	0.43	31.88
		slope + (1|zone)	54.88	4	1.99	0.16	13.48
	human + habitat	biomass + water + slope + elev + (1|zone)	52.90	7	0.00	0.59	31.88
		land use + biomass + water + slope + elev + (1|zone)	55.17	8	2.28	0.19	32.94

Akaike's information criterion corrected (AICc), number of parameters (k), difference in AIC between the model with the lowest AIC (dAIC), AICc weights (wAIC), percentage deviance explained (DEV). The most parsimonious models are shaded in grey. *Habitat variables* (habitat): slope in degrees from horizon (slope); distance (km) to closest permanent water (water); relative biomass (biomass); and elevation in metres above sea level (elev). *Human variables* (human): grazing land or non-grazing land (land use); and number of years the land burnt from 1997-2010, in the late dry season (fire). Köppen climate zone was modelled as a random effect (zone). Only models with wAIC > 0.1 are shown.

Chapter IV: Fifteen microsatellite loci for use in non-invasive sampling studies of antilopine wallaroo (*Macropus antilopinus*)

Antilopine wallaroo ©wildlifetourism
http://wildlifetourism.org.au/experiencing-our-wildlife/wildlife-tours/wildlife-travels-in-australia/rootourism-kangaroo-trail-australia/

Statement of authorship

Fifteen microsatellite loci for use in non-invasive sampling studies of antilopine wallaroo (*Macropus antilopinus*)

Accepted by *Australian Journal of Zoology* Nov 2013

JESSICA WADLEY (Candidate)
Performed DNA extractions, PCR amplifications, and sequencing, selected microsatellite loci from 454 data, conducted downstream processing and analysis of loci, interpreted the results, designed multiplex, created tables, and wrote the paper.

I hereby certify that the statement of contribution is accurate

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JEREMY AUSTIN
Helped design study, gave advice on laboratory work and edited manuscript

I hereby certify that the statement of contribution is accurate

Signed…… Date…..18/12/2013…..

MICHAEL GARDNER
Assisted in 454 analysis and microsatellite selection

I hereby certify that the statement of contribution is accurate

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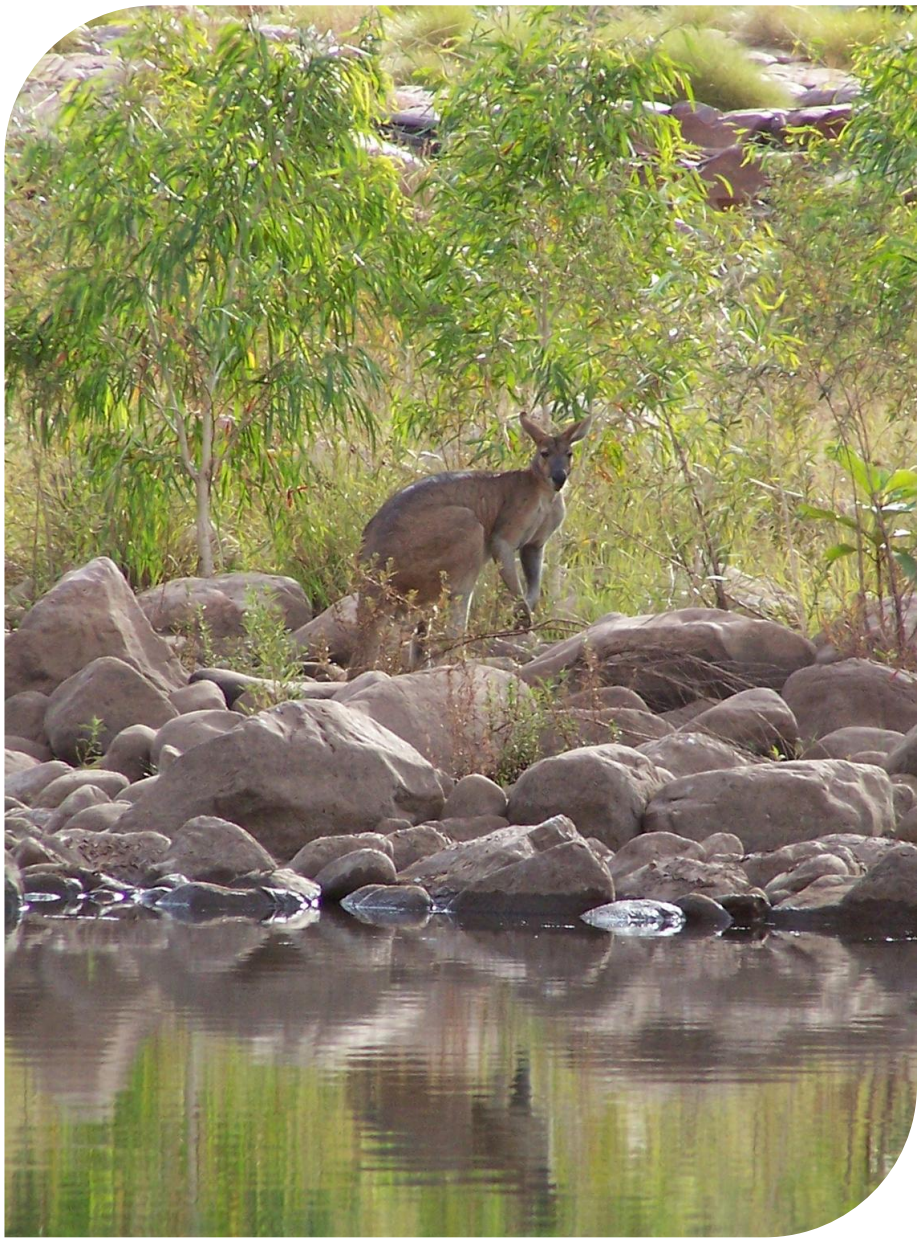
DAMIEN FORDHAM
Helped design study and edited manuscript

I hereby certify that the statement of contribution is accurate

Signed... Date…..18/12/2013…..

Wadley, J.J., Austin, J.J., Gardner, M.G. & Fordham, D.A. (2013) Fifteen microsatellite loci for use in non-invasive sampling studies of the antilopine wallaroo (Macropus antilopinus).
Australian Journal of Zoology, v. 61(5), pp. 399-401

Chapter V: Low genetic diversity and limited population structure in a large, vagile macropod (antilopine wallaroo; *Macropus antilopinus*) from Australia's tropical savannahs



Antilopine wallaroo ©Jessica Wadley

Statement of authorship

Low genetic diversity and limited population structure in a large, vagile macropod (antilopine wallaroo; *Macropus antilopinus*) from Australia's tropical savannahs

Text in manuscript in preparation for *Ecology and Evolution*

JESSICA WADLEY (Candidate)

Performed DNA extractions, PCR amplifications, RFLP species identification plus downstream processing and analysis on all samples, interpreted the data, created figures and tables, and wrote the paper.

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DAMIEN FORDHAM

Helped design study and edited manuscript

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Signed…… Date…..18/12/2013…..

Low genetic diversity and limited population structure in a large, vagile macropod (antilopine wallaroo, *Macropus antilopinus*) from Australia's tropical savannahs

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Abstract

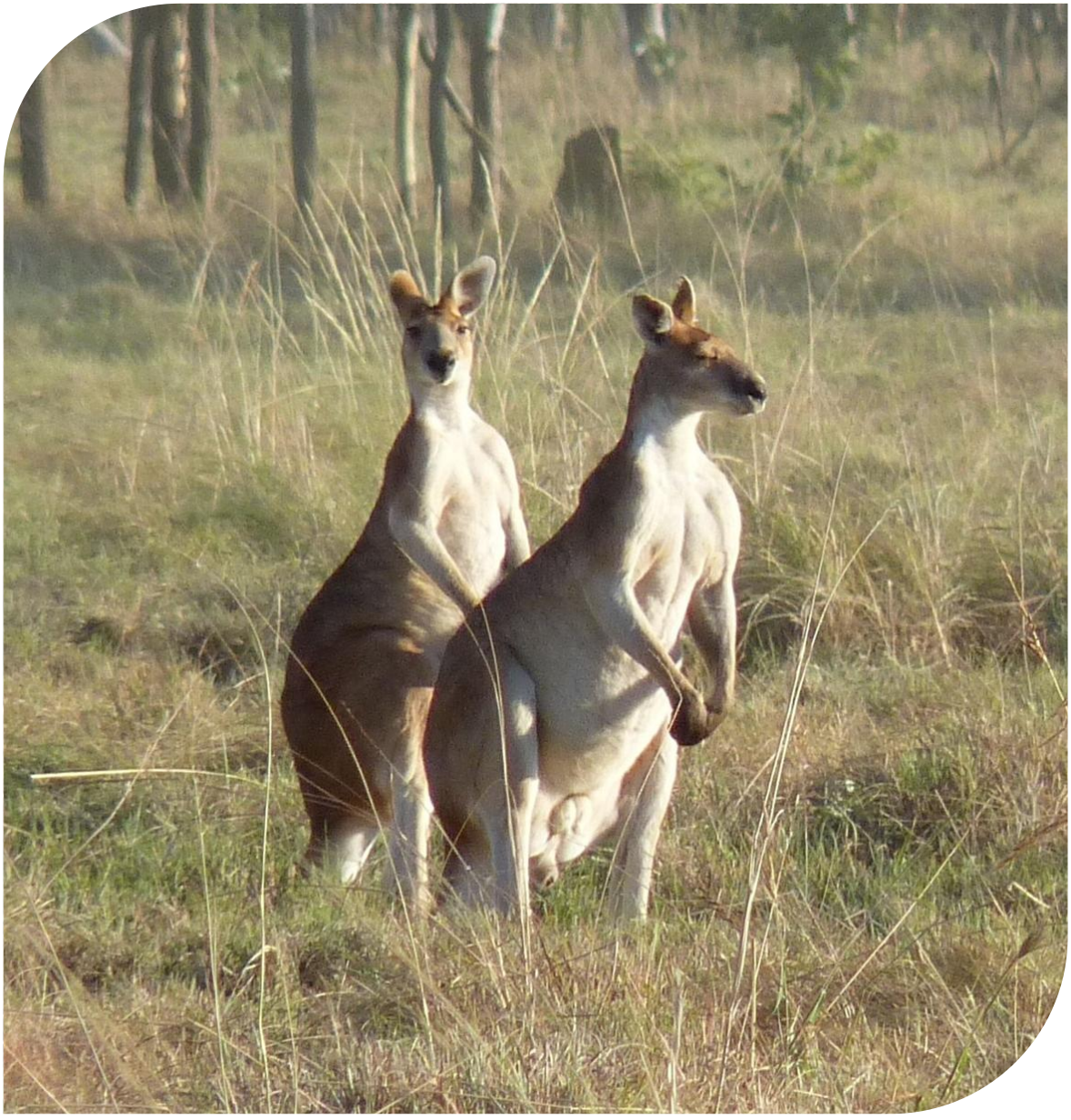
Genetic studies of macropod populations have shown that relatively abundant or widespread vagile species have high levels of genetic diversity and limited structuring. We used 15 microsatellite markers to investigate genetic diversity, population structure and gene flow among a previous un-assessed large macropod, *Macropus antilopinus*. Two distinct populations from the latitudinal extremes of the species continuous north-eastern Australian distribution were identified. Unlike other large vagile mammals, *M. antilopinus* displays low genetic diversity and lack of dispersal even in continuous habitat.

Keywords

Kangaroo, macropod, marsupial, microsatellite loci, population genetics

Wadley, J.J., Fordham, D.A. & Austin, J.J. (2013) Low genetic diversity and limited population structure in a large, vagile macropod (antilopine wallaroo, Macropus antilopinus) from Australia's tropical savannahs.
Ecology and Evolution, (in print)

Chapter VI: Phylogeography of the antilopine wallaroo (*Macropus antilopinus*) across northern Australia



Antilopine wallaroos © NTbirds
http://ntbirds.com.au/communities/5/004/011/458/375/images/4595470220.jpg

Statement of authorship

Phylogeography of the antilopine wallaroo (*Macropus antilopinus*)

Text in manuscript in preparation for *Journal of Zoology*

JESSICA WADLEY (Candidate)

Performed DNA extractions, RFLP species identification, PCR amplifications, and sequencing, plus downstream processing and analysis on all samples, interpreted the data, created figures and tables, and wrote the paper.

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DAMIEN FORDHAM

Helped design study and edited manuscript

I hereby certify that the statement of contribution is accurate

Signed... Date…..18/12/2013…..

VICKI THOMSON

Assisted in genetic analysis, interpretation of results and edited manuscript

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JEREMY AUSTIN

Helped design study, gave advice on laboratory work and edited manuscript

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Phylogeography of the antilopine wallaroo (*Macropus antilopinus*) across northern Australia

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Abstract

The tropical monsoon region of northern Australia represents a unique biome comprising a range of habitats and natural landscapes that are not heavily altered by human impacts. High species diversity and endemicity characterise the region and range boundaries of many species are often concordant, suggesting vicariant biogeographic processes have shaped present day biodiversity. The antilopine wallaroo (*Macropus antilopinus*) is a wide-spread and vagile macropod which occurs naturally across the tropical savannahs of northern Australia. The distribution of *M. antilopinus* is marked by a break in the species' range between Queensland and the Northern Territory at the base of the Gulf of Carpentaria, coinciding with the Carpentarian barrier. This area contains unsuitable habitat for *M. antilopinus*, which may be preventing dispersal and gene flow. A lack of gene flow could have important implications for taxonomy, management and conservation of *M. antilopinus*. We used mitochondrial DNA sequence data to examine phylogeographic patterns, infer the biogeographic barriers associated with these patterns, and estimate the timing of divergences across northern

Australia. MtDNA diversity revealed an unexpectedly complex evolutionary history. Across the monsoon tropics of northern Australia, *M. antilopinus* show three distinct, but relatively young, mtDNA clades, with limited evidence for a fourth, much older lineage. The three clades can be considered geographically distinct with the intervening areas possibly representative of barriers to dispersal for this species (e.g. Carpentarian barrier). Insufficient sample size for the fourth lineage prevents identification of its range or association with known barriers. The divergence time estimates obtained for these clades can be linked to environmental changes occurring across northern Australia during the Late Pleistocene. These genetic and geographical patterns highlight the importance of investigating genetic variation across species distributions and integrating this information into species' conservation management.

Keywords

Biogeographic barriers, Carpentarian, divergence, genetics, macropod, marsupial, tropical savannah

Introduction

The tropical monsoon region of northern Australia represents a unique biome comprising a range of habitats and natural landscapes that are relatively intact and unaffected by recent human activities (Potter *et al.* 2012). The regional climate is characterised by dry winters and wet summers, which has strongly affected the distribution of plant and animals species (Bowman *et al.* 2010). The range boundaries of many of these species are often concordant, suggesting vicariant barriers have played a major role in influencing the biogeography in the area (Rollins *et al.* 2012). To date phylogeographic analyses (reviewed in (Bowman *et al.* 2010)) have supported inter- and intra-specific vicariance for many species in response to northern Australian barriers.

The antilopine wallaroo (*Macropus antilopinus*) occurs naturally across the tropical savannahs of northern Australia. It exhibits a suite of unique biological traits, not found in other macropods, such as a bulbous nose which assists with cooling when panting (Ritchie 2010). Although *M. antilopinus* is considered by the IUCN to be a 'species of least concern' (Woinarski *et al.* 2008), it is potentially vulnerable to extinction due to its dependence on tropical grasslands and its vulnerability to climate change. Climate change poses a significant risk to the persistence of *M. antilopinus* due to the species' relatively restricted distribution compared to other macropods, high dependence on permanent water sources and seasonal breeding patterns (Ritchie 2007).

Phylogeography can provide information on the number and geographic distribution of populations within a species range thus defining important units for conservation (evolutionary significant units (ESUs) and management units (MUs) as defined by Moritz (1994)), as well as identifying the relative roles of contemporary verses historical processes that have shaped within-species diversity (Avise 2000). The distribution of *M. antilopinus* is

marked by a break in the species' range between Queensland (QLD) and the Northern Territory (NT) at the base of the Gulf of Carpentaria (Figure 1). The Gulf of Carpentaria and the extensive arid lowland plains of the Gulf Country form a geographic barrier, known as the 'Carpentarian Barrier' or 'Carpentarian Gap' which divides Cape York Peninsula from the Top End (Braby 2008). This barrier is generally assumed to have arisen as a result of fluctuations in sea level over the Pleistocene as well as a steep climate and habitat gradient across this area during the last glacial maximum (Cracraft 1986; Lee & Edwards 2008). The Carpentarian barrier is approximately 150-km wide and is a semiarid region extremely poor in vegetation (Lee & Edwards 2008).

Phylogeographic studies on a range of taxa confirm the importance of the Carpentarian barrier in dividing western and eastern populations (Jennings & Edwards 2005; Braby 2008; Lee & Edwards 2008; Toon *et al.* 2010) with estimated divergence times suggesting mid-late Pleistocene divergence. Prior to the most recent marine inundation of the Gulf of Carpentaria (~ 10,000 years ago (Chivas *et al.* 2001)), gene flow between populations of *M. antilopinus* in the NT and QLD may have occurred widely across the Carpentarian plain. Following marine inundation, the only possible connection between the two populations was across the Carpentarian barrier. However, the Carpentarian plains contain unsuitable habitat for *M. antilopinus*, which may have prevented subsequent dispersal and gene flow between QLD and NT populations (Ritchie *et al.* 2008). A lack of gene flow could have important implications for taxonomy, management and conservation of *M. antilopinus* populations.

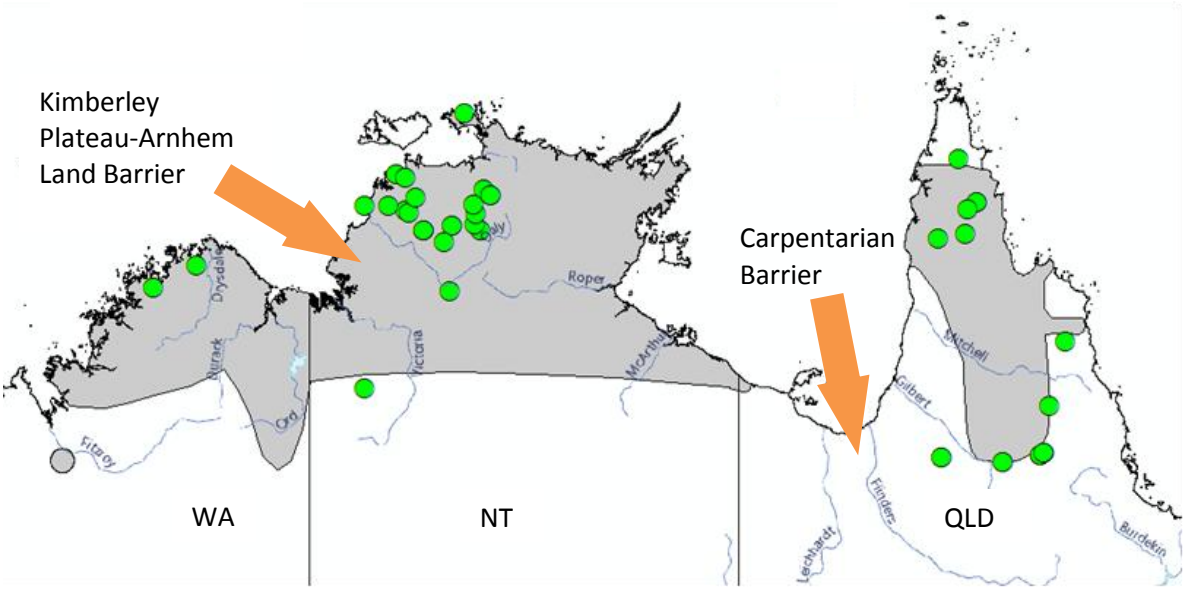


Figure VI-1.Current distribution of *Macropus antilopinus* across northern Australia showing sample locations, major rivers and biogeographic barriers. The 50 sampling locations are shown in green. The current distribution is in grey clearly showing the break in range at the Carpentarian barrier (orange arrow) between Queensland (QLD) and the Northern Territory (NT). The Kimberly-Plateau-Arnhem Land barrier is also shown by an orange arrow between Western Australia (WA) and the NT. The major rivers are shown in blue with the Ord, Victoria and Daly rivers all being implicated in the Kimberly-Plateau-Arnhem Land barrier. For detailed locality data see Supplementary Table S1.

Northern Australia also contains a suite of western barriers that may have affected gene flow among *M. antilopinus* populations in the NT and Western Australia (WA). Ford (1978) was the first to propose a 'minor' biogeographic barrier splitting Arnhem Land and the Kimberley, referred to as the Bonaparte barrier. More recently additional, geographically proximate barriers have been identified: the 'Ord Arid Intrusion' (Bowman *et al.* 2010), 'Bonaparte Gap' (Toon *et al.* 2010), Daly River Drainage Barrier (Potter *et al.* 2012), and the Victoria River Drainage Barrier (Joseph & Omland 2009). Eldridge *et al.* (2011) reviewed these barriers and suggested a single term - the Kimberley Plateau-Arnhem Land Barrier - be applied to the whole region, encompassing the four specific barriers. Arnhem Land and the Kimberley Plateau are areas of relatively high relief, rainfall, and more mesic woodland then the river catchments separating them (Daly, Victoria and Ord Rivers) possibly representing more ideal habitat for *M. antilopinus* (Ford 1978; Cracraft 1986).

As one of the few large terrestrial mammals restricted to the northern tropics, understanding the phylogeography of *M. antilopinus* is important to gain a broader understanding of historical biogeography in the region and the evolutionary processes shaping diversity. A previous study using mitochondrial DNA and a limited number of individuals revealed low levels of genetic divergence and no major phylogeographic break between the NT and QLD populations of *M. antilopinus* (Ritchie 2007). Here, we re-examine this result using additional sampling on either side of the Carpentarian barrier, and address the impact of the Kimberley Plateau-Arnhem Land Barrier. Specifically, we use mitochondrial DNA sequences to (i) determine phylogeographic patterns across northern Australia, (ii) infer the biogeographic barriers associated with these patterns, and (iii) estimate the timing of divergences.

Methods

Sample collection

A total of 89 *M. antilopinus* samples were obtained as DNA extracts from a previous study (Ritchie 2007), and as tissue and bone samples from museum collections . The bone samples included both bone and dry tissue remnants removed from within the nasal cavity of museum skulls. The total dataset was geographically sampled from QLD (n=29), the NT (n=44), a small number from WA (n=12) and four samples with unknown locality.

DNA extraction, amplification and sequencing

Three mtDNA fragments, 1,042 bp of NADH dehydrogenase subunit 2 (ND2), 1,146 bp of cytochrome b (cytb) and 609 bp of control region (CR) were targeted. These fragments have been widely used in macropod phylogenetics (Fumagalli *et al.* 1997; Bulazel *et al.* 2007; Phillips *et al.* 2013).

All extractions were performed with negative extraction controls to monitor for DNA contamination during the extraction procedures. In addition, all PCRs were performed with negative PCR controls to monitor for contamination during the amplification procedures. DNA was extracted from frozen tissue samples using a 'salting out' procedure (Nicholls *et al.* 2000). Tissue samples and DNA extracts were amplified and sequenced using primers; M1034 and M1035 for ND2; M441 and M442 for cytb; and L15999M and H16498M for CR (Table 1). Additional internal sequencing primers were also designed and used for the ND2 and cytb region; M461 and M440 for ND2; and M462 and M463 for cytb. Tissue sample and DNA extract PCRs were conducted in 25 µl volumes containing 2 µl of DNA, 1x HotMaster PCR Buffer (Eppendorf), 0.2 mM each dNTP, 0.4 µM of each primer and 0.5 U HotMaster *Taq* DNA Polymerase (Eppendorf). Thermocycling was conducted using 2 min enzyme activation at 94 °C followed by 35 cycles of 94 °C for 20 sec, 55 °C for 10 sec and 65 °C for 60 sec, with

a final extension step of 65 °C for 10 min. PCR products were purified using AMPure (Agencourt) as per manufacturer's instructions and Sanger sequenced with BigDye v3.1 chemistry (Life Technologies) in 1/16 reactions using the original PCR primers and the additional internal sequencing primers. Sequencing reactions were purified using CleanSEQ (Agencourt) as per manufacturer's instructions and run on an ABI 3130XL Genetic Analyzer.

Table VI-1. Primers used to amplify and sequence modern tissue samples and DNA extracts.

Primer name	Pseudonym	Sequence 5' - 3'	Region	Reference
External				
M441	Ma_cytb_Mr1_F	CATTTTAGTATGGACTCTAACCATAACC	cytb	(Bulazel *et al.* 2007)
M442	Ma_cytb_Mr2_R	AGGGTGTTATACCTTCATTTTTGG	cytb	(Bulazel *et al.* 2007)
M1034	Ma_ND2_F	CCCTTCCCATACTAATGTC	ND2	Designed in this study
M1035	Ma_ND2_R	GATTTGCGTTCGGATGA	ND2	Designed in this study
L15999M	Ma_CR_L15999M_F	ACCATCAACTCCCAAAGCTGA	CR	(Fumagalli *et al.* 1997)
H16498M	Ma_CR_H16498M_R	CCTGAAGTAGCAACCAGTAG	CR	(Fumagalli *et al.* 1997)
Internal				
M462	Ma_cytb_IntF	GACAAAGCCACCCTCACACGCT	cytb	Designed in this study
M463	Ma_cytb_IntR	TGTTTCGTGTAGGAATAGGAGGTGGA	cytb	Designed in this study
M461	Ma_ND2_IntF3	AACAATGCTCGGAGGCTGAG	ND2	Designed in this study
M440	Ma_ND2_IntR	AGGCTAGGATTTTTCGTAGGTG	ND2	Designed in this study

DNA was extracted from bone samples using a Qiagen DNeasy blood and tissue kit with the following modification; the amount of ATL, AL and ethanol added were doubled. Bone extractions were conducted in a clean-room laboratory in a separate building to avoid contamination from modern DNA and PCR products. Bone extracts underwent amplification and sequencing using species identification primers designed in Wadley *et al.* (2013) to determine their successful extraction and species identification before being used in additional PCRs. Bone extracts were amplified and sequenced with a number of short overlapping fragments (Table 2). PCRs performed with DNA extracts from bones samples were conducted

in 25 µl volumes containing 3 µl of DNA, 1x High Fidelity PCR Buffer (Invitrogen), 0.25 mM each dNTP, 2 mM $MgSO_4$, 1 mg/ml RSA, 0.4 µM of each primer and 1 U Platinum® *Taq* High Fidelity (Invitogen). Thermocycling was conducted using 2 min enzyme activation at 94 °C followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 30 sec, with a final extension step of 68 °C for 10 min. PCR products were then purified using Millipore MultiScreen PCR_{384}Filter Plates (Millipore) and directly sequenced on an AB 3730*xl* DNA Analyzer (Applied Biosystems) at the Australian Genome Research Facility (AGRF), Adelaide.

Table VI-2. Primers used to amplify and sequence museum bone samples.

Primer name	Pseudonym	Sequence 5' - 3'	Region	Reference
A2086	Ma_cytb_Mr1_F1	CATTTTAGTATGGACTCTAACCATAACC	cytb	(Bulazel *et al.* 2007)
A2087	Ma_Cytb_mus_intR1	CGTCTCGGCAGATATGGGCA	cytb	Designed in this study
A2088	Ma_Cytb_mus_intF2	ATACCTCAGACACCCTAACAG	cytb	Designed in this study
A2089	Ma_Cytb_mus_intR2	GACAGGAGGTTTGTGATTACGG	cytb	Designed in this study
A2090	Ma_Cytb_mus_intF3	TGTCCTACCATGAGGACAAAT	cytb	Designed in this study
A2091	Ma_Cytb_mus_intR3	GATGGTATAATATGGGTGGAATGG	cytb	Designed in this study
A2092	Ma_Cytb_mus_intF4	TCCGGAATTAACCCCGACTC	cytb	Designed in this study
A2093	Ma_Cytb_mus_intR4	AAGATGGATGCTAGCAGAGC	cytb	Designed in this study
A2094	Ma_Cytb_mus_intF5	CCCTAACAAACTAGGAGGAGT	cytb	Designed in this study
A2095	Ma_Cytb_mus_intR5	GTATGTAGTTTTCAAATAATCCGGC	cytb	Designed in this study
A2096	Ma_ND2_mus_intF1	TCAGCCTCTTCCTAGGCACATCCC	ND2	Designed in this study
A2097	Ma_ND2_mus_intR1	GGAAGGGGGCCAGGCCTAGTTT	ND2	Designed in this study
A2098	Ma_ND2_mus_intF2	ACAGCCTCAGTCCTAATAACCCTAGCA	ND2	Designed in this study
A2099	Ma_ND2_mus_intR2	TCCAGCCTATATGGGCGATGGAA	ND2	Designed in this study
A2100	Ma_ND2_mus_intF3	TGCTCGGAGGCTGAGGAGGC	ND2	Designed in this study
A2101	Ma_ND2_mus_intR3	TGGTATGAAGCCGGTTAGAGGAGGA	ND2	Designed in this study
A2102	Ma_ND2_mus_intF4	CCTCCTCACACTTCTATCTCTAGGCGG	ND2	Designed in this study
A2103	Ma_ND2_mus_intR4	GGGGTGTTAGTGGGAGTAGGAGGG	ND2	Designed in this study
A2104	Ma_CR_L15999M_F1	ACCATCAACTCCCAAAGCTGA	CR	(Fumagalli *et al.* 1997)
A2105	Ma_CR_mus_intR1	TGAGGTTTAATGTATTTAGTAATGTGTGA	CR	Designed in this study
A2106	Ma_CR_mus_intF2	GCAATACATAGAATTAATGGTAACTAAGA	CR	Designed in this study
A2107	Ma_CR_mus_intR2	GTAGTATGTCATTATAGATACGCTAGT	CR	Designed in this study
A2108	Ma_CR_mus_intF3	ACCAAAACGTGCATAAATGACT	CR	Designed in this study
A2109	Ma_CR_mus_intR3	ATGGGCCTGCTCTGAAGGAT	CR	Designed in this study

Genetic diversity

Sequence chromatograms were edited and individual sample contigs assembled using Sequencher 4.7 (Gene Codes Corporation) and sequences aligned using Geneious Pro 5.6.4 (Biomatters). The three mtDNA regions were concatenated to create a dataset for analysis of all *M. antilopinus* samples, and where needed, one *M. robustus* sample was included as an outgroup. The computer program ARLEQUIN version 3.5 (Excoffier & Lischer 2010) was used to calculate haplotype diversity (*h*) and nucleotide diversity (π), and to determine the number of variable sites, transitions, transversions and haplotypes. Mantel tests and Principle Coordinate Analysis (PCoA) were conducted in GenAlEX version 6.5 (Peakall & Smouse 2012) based on genetic and geographic distances between samples. A hierarchical AMOVA analysis of populations was performed in GenAlEX to examine the distribution of variation and differential connectivity among populations (PhiPT), regions (PhiRT), and populations within regions (PhiPR). A variety of population hypothesis were tested based on results from phylogenetic analyses.

Phylogenetic analyses

The concatenated dataset was used in the reconstruction of a phylogenetic tree of *M. antilopinus* to include all evolutionary information available. PARTITIONFINDER version 1.1.0 (Lanfear *et al.* 2012) was run to determine the appropriate models of sequence evolution for the phylogenetic analysis. Reconstruction of a phylogenetic tree was based on Bayesian analysis and employed by the software MRBAYES version 3.2 (Ronquist *et al.* 2012). Four independent runs of Markov chain Monte Carlo analysis were performed with 2,500,000 cycles sampling every 250 cycles. Four chains (1 cold and 3 heated) were used with a temperature of 0.1. The burnin was set to 25 % and branch length information was saved on the sampled trees. Summary statistics were produced for all sampled trees and a consensus

tree produced containing the branch lengths and interior nodes labelled with support values. The consensus tree was then visualised using FIGTREE version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Haplotype network

NETWORK version 4.6.1.1 (Bandelt *et al.* 1999) was used to create intraspecific median-joining (MJ) networks to visualise evolutionary relationships between haplotypes. The data matrix used in the NETWORK analysis included the concatenated nucleotide sequences of the three mtDNA regions for the 49 *M. antilopinus* specimens successfully sequenced for all three fragments.

Demography

Historical demographic patterns were inferred using Tajima's D (Tajima 1989) and Fu's Fs statistics (Fu 1997) to test for deviations from neutrality, and by analysis of mismatch distributions with 100 bootstrap replicates in ARLEQUIN. The mismatch analysis compares distributions of pairwise nucleotide differences to permuted distributions under the null hypothesis of demographic expansion and spatial expansion. The overall validity of the estimated expansion models was evaluated by the tests of raggedness index (HRI) (Harpending 1994) and the sum of squared differences (SSD) (Schneider & Excoffier 1999). The SSD at a level of $P \leq 0.05$ was taken as evidence for departure from sudden population or spatial expansion models. Low HRI values (< 0.1) indicate a significant fit between the observed and the expected distributions.

Divergence time and mutation rate estimates

We estimated divergence times of *M. antilopinus* populations using a Bayesian phylogenetic approach, implemented in BEAST 1.7.5 (Drummond *et al.* 2012). Analyses were performed

using models of nucleotide substitution previously estimated in PARTITIONFINDER. The divergence estimate of the *M. robustus* and *M. antilopinus* species split was taken from Meredith *et al.* (2008) in order to calibrate the phylogenetic tree. The analysis was run using a constant population size and strict molecular clock. Ten independent MCMC chains were run for 10 million generations sampling every 10000 generations, and a 10% burn-in was removed from the posterior samples.

Results

Partial CR sequences (597 bp) were obtained from 57 *M. antilopinus* samples. The ND2 region (945 bp) and cytb region (1104 bp) were sequenced for 52 and 51 samples respectively. Due to all fragments being mitochondrial in origin and inherited as a single locus, the samples with positive results for all three regions were combined and aligned (*M. antilopinus* n=50; QLD n=23, NT n=25, and WA n=2) (Figure 1, Supplementary Table S1). However, sample 11236 (from the NT) was removed from some analyses due to being a single sample with a large number of unique variable sites (62) not found in any other *M. antilopinus* samples (resulting in, n=24 for NT). The remaining 49 *M. antilopinus* sequences contained 74 variable sites that defined 40 distinct mtDNA haplotypes in the 2646 bp sequence analysed. Transitions comprised 72 of the observed nucleotide substitutions while transversions accounted for three of the sites (there was one multivariable site). There were also two indels observed in the control region.

Phylogenetic relationships

The phylogenetic tree (Figure 2) and network (Figure 3) show samples falling into three clades, a combined Northern Territory and Western Australian group (referred to as NT1/WA), a second Northern Territory group (NT2), and a Queensland group (QLD). Sample 11236 forms a basal lineage to the three main clades. The WA samples, although containing a higher level of variation compared to NT1 samples, fall within the diversity of the NT1 samples. These groupings are also supported by significant population pairwise phiPTs > 0.5 for all comparisons (Table 3), a high degree of differentiation (coordinate percentage) and clear groupings in the PCoA (Figure 4) and > 32 % variance among these groups determined in the AMOVA analysis (Table 4). The higher divergence between NT1 and NT2 compared to NT2 and QLD was surprising, with no clear interpretation. The exact locations of these NT1

and NT2 samples, revealing the lack of geographic overlap between the clades is shown in Figure 5. Another interesting result was found within the NT1/WA grouping. A slight segregation was seen in the PCoA (Figure 4) between the two WA samples and the lowest latitude NT sample (ABTC27780). The differentiation between these three samples and the remaining NT1 samples may represent a possible weak biogeographic barrier.

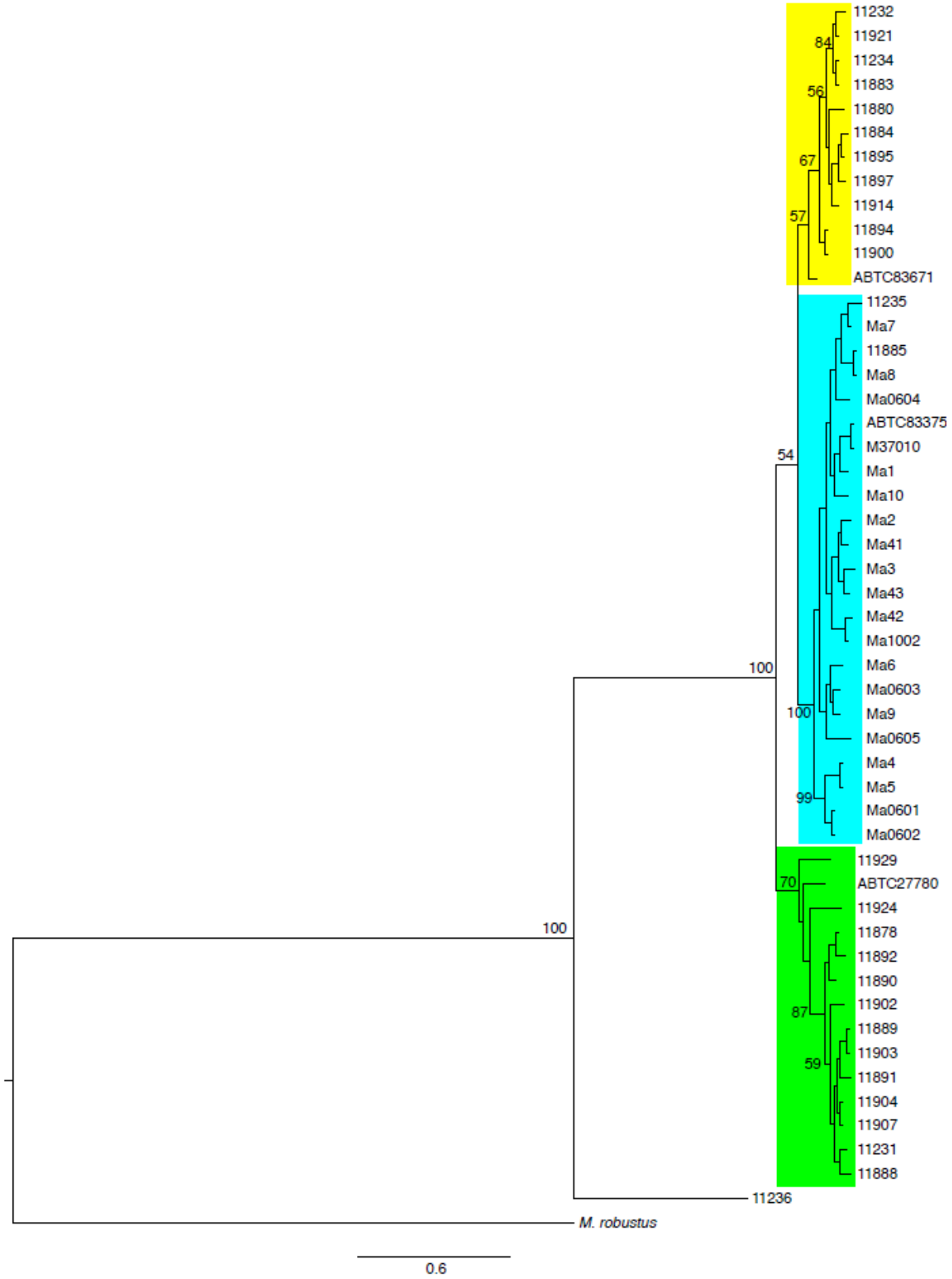


Figure VI-2. MrBayes phylogenetic tree of concatenated dataset for 50 *Macropus antilopinus* samples (including 11236) and one outgroup (*M. robustus*). Probability is shown as branch labels. Highlighted aqua clade contains the QLD samples, yellow contains NT2, and green includes the WA and NT1 samples.

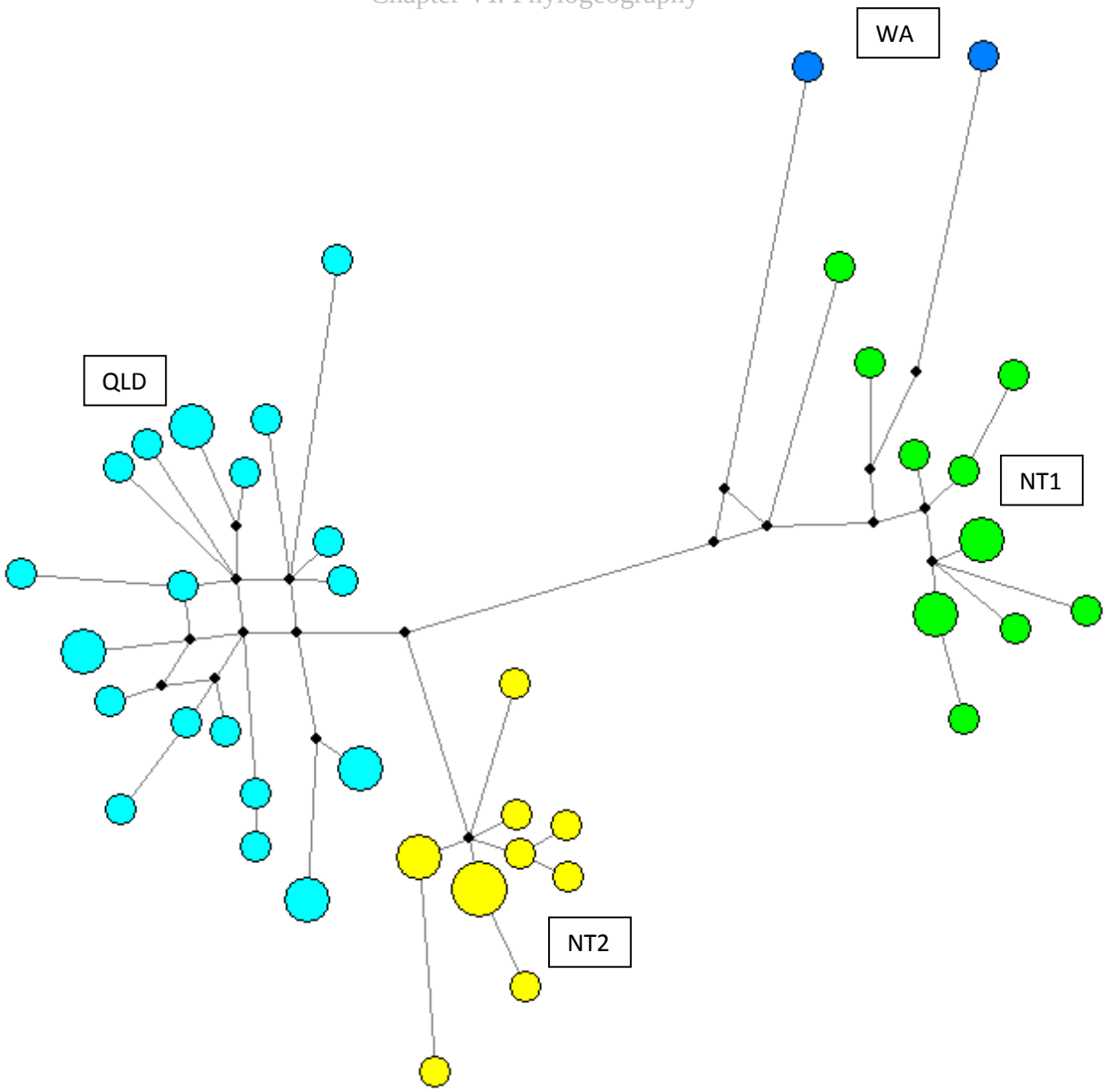


Figure VI-3. Intraspecific median-joining (MJ) network showing the evolutionary relationships between haplotypes of *Macropus antilopinus*, excluding sample 11236. QLD samples are shown in aqua, NT2 in yellow, NT1 in green and WA in dark blue. Circle size is scaled to frequencies of haplotypes and branches scaled to the number of mutational steps.

Table VI-3. Pairwise population PhiPT values for *Macropus antilopinus* PhiPT values are below the diagonal. Probability, P(rand >= data) based on 999 permutations is shown above the diagonal.

	WA and NT1	**NT2**	**QLD**
WA and NT1	0.000	0.001	0.001
NT2	0.689	0.000	0.001
QLD	0.624	0.552	0.000

Table VI-4. AMOVA results for *Macropus antilopinus* populations.

Comparison groupings	**ϕ statistics**			**Percentage variation**		
	ϕSC (PhiPR) (pop within region)	ϕCT (PhiRT) (among region)	ϕST (PhiPT) (among pop)	among groups	among populations within groups	within populations
3 population hypothesis based on network						
(QLD) (NT2) (NT1 / WA) grouped by state location	0.47415**	0.32851**	0.64690**	32.85	31.84	35.31
3 population hypothesis based on PCoA						
(QLD) (NT2) (NT1 / WA) when 27780 grouped with WA samples	0.65234**	0.36770**	0.45017**	36.77	28.46	34.77
3 population hypothesis based on states						
(QLD) (NT) (WA)	0.69658**	-0.22333	0.62882**	-22.33	85.21	37.12
2 population hypothesis based on east west split						
(QLD / NT2) (NT1 / WA)	0.53495**	0.32884**	0.68787**	32.88	35.9	31.21
(QLD / NT2) (NT1 / WA) when 27780 grouped as WA	0.53045**	0.34992**	0.69475**	34.99	34,48	30.52

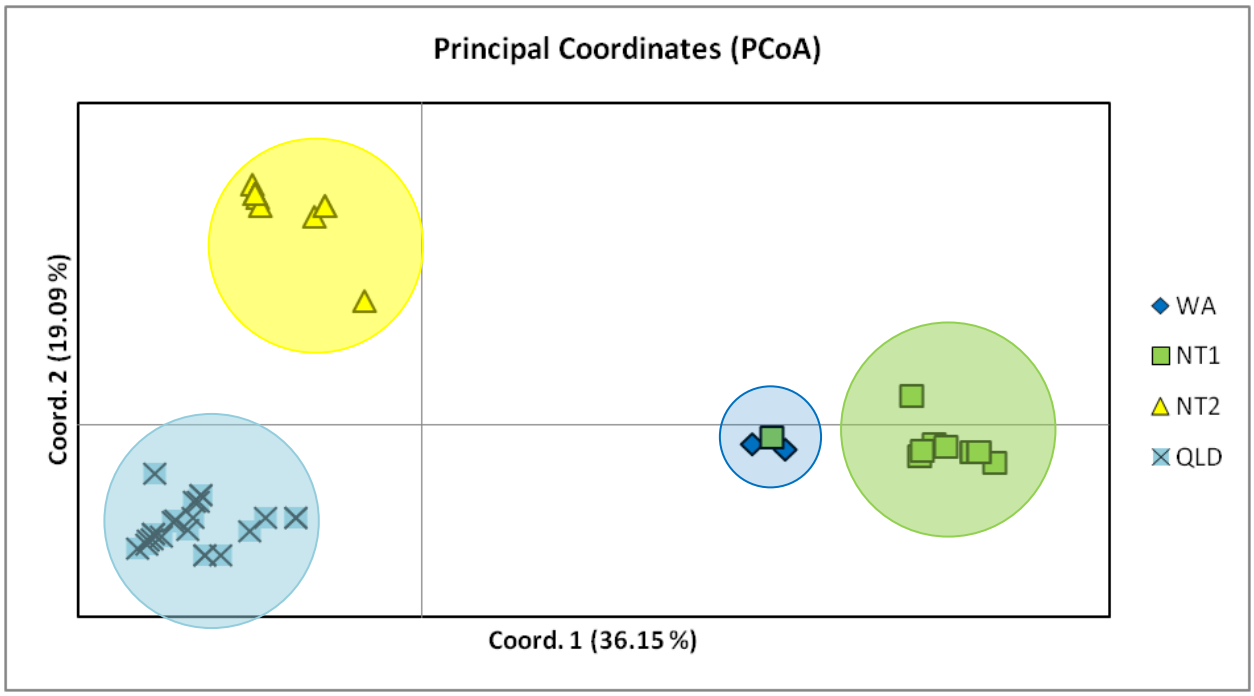


Figure VI-4. Principal Coordinates Analysis (PCoA) via covariance matrix with data standardization of genetic distance for *Macropus antilopinus* samples, excluding sample 11236. QLD samples are show as aqua crosses, NT2 as yellow triangles, NT1 as green squares and WA samples in dark blue diamonds.

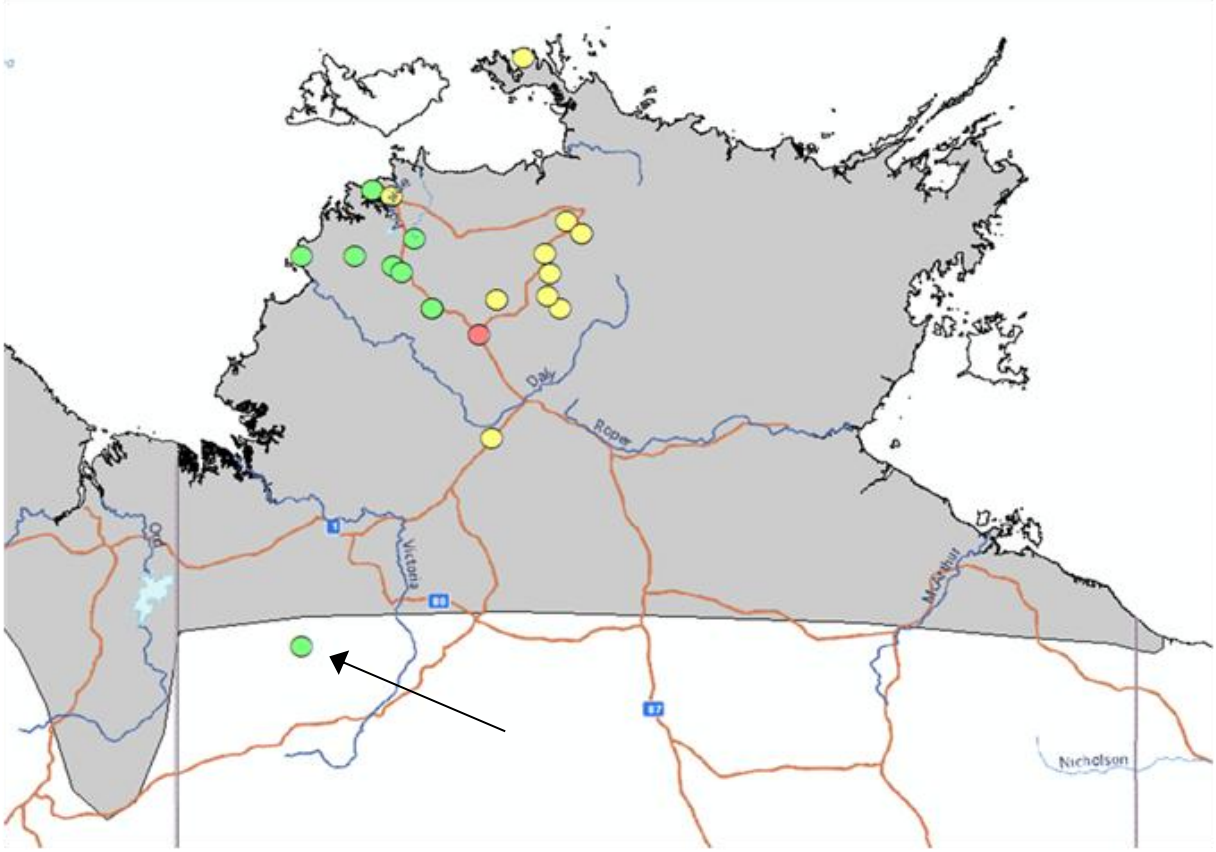


Figure VI-5. Map of the Northern Territory showing the location of *Macropus antilopinus* samples belonging to clades NT1 and NT2. NT1 samples are shown in green and NT2 samples shown in yellow. The red sample is the location of the divergent sample 11236. The arrow indicates the NT1 sample that groups with the WA samples from the PCoA (ABTC27780). Major rivers are shown in blue and highways in brown.

Estimates of time since divergence were calculated for each of the three clades. These estimates indicate that an initial split between the western (NT1 and WA) and eastern clades (NT2 and QLD) occurred approximately 52,000 years ago. The divergence between NT2 and QLD occurred 34,000 years ago. The split between all *M. antilopinus* samples and the divergent sample (11236) was estimated to be 210,000 years ago.

Demography

Tajima's D and Fu's Fs statistics show negative values in most population groupings with statistically significant results for the QLD, NT2, and combined NT1/WA populations only. Population groupings with non-significant neutrality statistics include: NT1 samples alone, NT1 and NT2 combined, combined QLD and NT2, and all samples as a global population (Table 5).

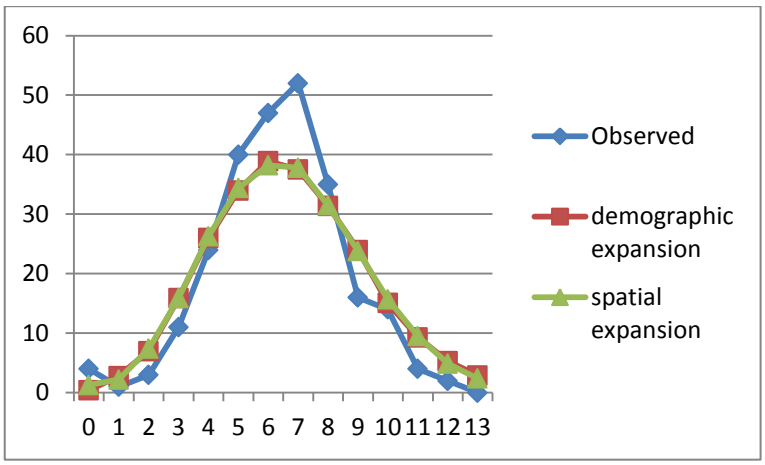
Table VI-5. Genetic diversity and historical demographic patterns of *Macropus antilopinus* populations and the global sample set from across northern Australia The greyed out hypothesis is supported by the phylogenetic tree and network.

Population		**Genetic diversity**		**Neutrality test**		**MD demographic expansion**		**MD spatial expansion**	
Name	**Size (number of haplotypes)**	**H**	**π**	**Tajima *D***	**Fu's Fs**	**SSD**	**HRI**	**SSD**	**HRI**
All (with 11236)	50 (41)	0.9918 ±0.0058	0.0056 ±0.0028	-1.8433*	-18.7108*	0.0097	0.0101	0.0149	0.0101
All (excluding 11236)	49 (40)	0.9915 ±0.0060	0.0046 ±0.0023	-0.9840	-21.5719*	0.0100	0.0109	0.0142*	0.0109
3 population hypothesis based on network									
QLD	23 (19)	0.9842 ±0.0170	0.0024 ±0.0013	-1.2571	-9.8052*	0.0072	0.0209	0.0072	0.0209
NT2	12 (9)	0.9394 ±0.0577	0.0012 ±0.0008	-1.4384	-3.6439*	0.0286	0.1267	0.0282	0.1267
NT1andWA (excluding 11236)	14 (12)	0.9780 ±0.0345	0.0027 ±0.0015	-1.3861	-3.9287*	0.1281*	0.0372	0.0272	0.0372
4 population hypothesis (27780 grouped with WA) based on PCoA									
QLD	23 (19)	0.9842 ±0.0170	0.0024 ±0.0013	-1.2571	-9.8052*	0.0072	0.0209	0.0072	0.0209
WA (with 22780)	3 (3)	1.0000 ±0.2722	0.0053 ±0.0041	0.0000	1.4939	0.2003	0.6667	0.2003	0.6667
NT1 (removed 22780 and excluding 11236)	11 (9)	0.9636 ±0.0510	0.0014 ±0.0009	-1.2890	-3.7134	0.0221	0.0707	0.0221	0.0707
NT2	12 (9)	0.9394 ±0.0577	0.0012 ±0.0008	-1.4384	-3.6439*	0.0286	0.1267	0.0282	0.1267
4 population hypothesis									
QLD	23 (19)	0.9842 ±0.0170	0.0024 ±0.0013	-1.2571	-9.8052*	0.0072	0.0209	0.0072	0.0209
WA	2 (2)	1.000 ±0.000	0.0060 ±0.0062	0.0000	2.7726	NA	NA	NA	NA
NT1 (excluding 11236)	12 (10)	0.9697 ±0.0443	0.0019 ±0.0011	-1.5552	-3.7113*	0.0211	0.0551	0.0207	0.0551
NT2	12 (9)	0.9394 ±0.0577	0.0012 ±0.0008	-1.4384	-3.6439*	0.0286	0.1267	0.0282	0.1267
3 population hypothesis based on state									
QLD	23 (19)	0.9842 ±0.0170	0.0024 ±0.0013	-1.2571	-9.8052*	0.0072	0.0209	0.0072	0.0209
combined NT (excluding 11236)	24 (19)	0.9783 ±0.0187	0.0041 ±0.0021	-0.0745	-5.0909*	0.0359	0.0407	0.0450*	0.0407
WA	2 (2)	1.000 ±0.000	0.0060 ±0.0062	0.0000	2.7726	NA	NA	NA	NA
2 population hypothesis									
QLDandNT2	35 (28)	0.9866 ±0.0102	0.0032 ±0.0017	-1.1647	-14.8526*	0.0146	0.0200	0.0173*	0.0200
NT1andWA (excluding 11236)	14 (12)	0.9780 ±0.0345	0.0027 ±0.0015	-1.3861	-3.9287*	0.1281*	0.0372	0.0272	0.0372

Mismatched distribution (MD), haplotype diversity (H), nucleotide diversity (π), raggedness index (HRI) and the sum of squared differences (SSD).

Overall, the mismatch distributions supported population expansions as expected under sudden (recent) demographic expansion and spatial expansion models (range expansion with high levels of migration between neighbouring populations) as seen in the small SSD values and a lack of significant evidence against the expansion models in most populations (Table 5). One combined population, NT1/WA, determined via the network analysis, produced a significant result against the sudden expansion model, indicating a departure from this demographic model (Figure 6). Three of the possible populations with significant evidence against the spatial expansion model included the species in entirety, and the NT1/NT2 and QLD/NT2 populations, most likely due to the higher variation introduced when these samples were grouped inappropriately. The small HRI values as well as the lack of significance for any groupings, reflects the unimodal shape of the mismatch distributions for the species shown in Figure 6.

a



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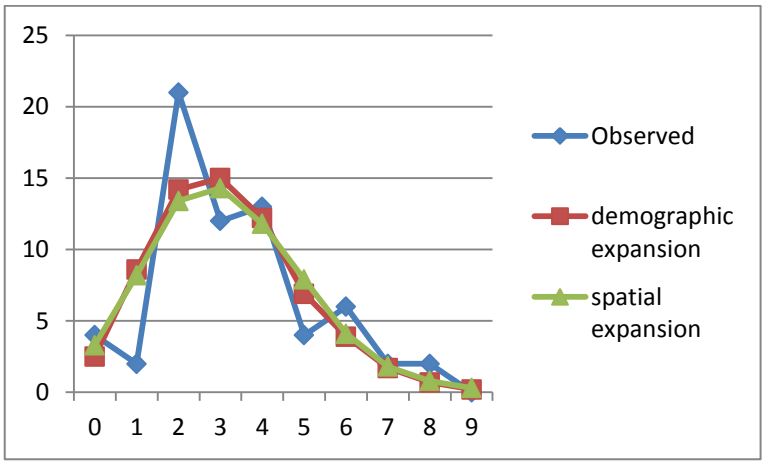

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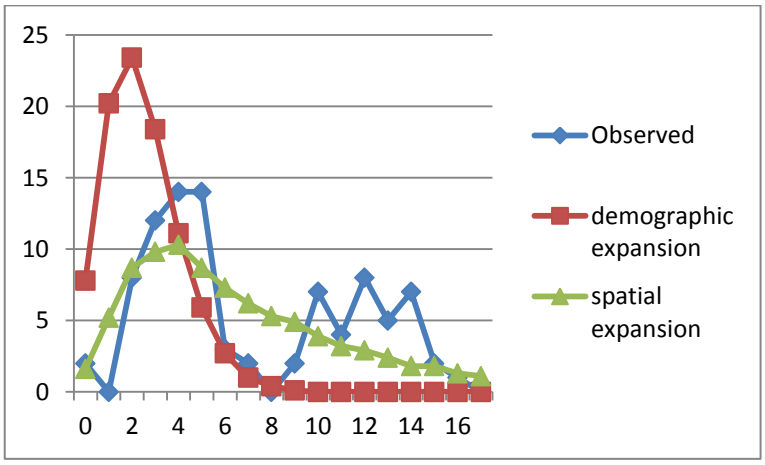

Figure VI-6. Mismatch distributions for *M. antilopinus* populations. a = QLD, b = NT2 c = NT1/WA.

Discussion

Our examination of mtDNA phylogeography in *Macropus antilopinus*, a wide-spread and vagile Australian macropod, has revealed an unexpectedly complex evolutionary history. Across the monsoon tropics of northern Australia, three shallow, but distinct mtDNA clades are identified, with a single sample (11236) representing a fourth, basal lineage.

One outlier sample from central NT (11236) contained a large number of unique mutations relative to, and fell basal to, the remaining samples with a divergence time of 210,000 years ago. A number of factors suggest that mtDNA sequence obtained from 11236 are authentic and do not represent nuclear copies or contamination. Firstly, two additional *M. antilopinus* samples, sequenced only for the ND2 and cytb fragments, also fell basal and grouped with 11236 in the preliminary *Macropus* phylogeny (Appendix 1). Secondly, all three samples are museum bone samples that were amplified with multiple sets of primers. All fragments show unique substitutions relative to the remaining *M. antilopinus* samples, so the resulting sequences are unlikely to be the product of nuclear copies of all three mitochondrial genes (numts). Thirdly, when comparing 11236 to one of the NT1 samples the variable sites found within each fragment included 21 in the cytb gene that were synonymous 3rd codon transitions, one a synonymous 1st codon transition and three non-synonymous 1st codon transitions. Similarly the ND2 fragment contained 16 synonymous 3rd codon transitions, one synonymous 1st codon transition and one non-synonymous 2nd codon transition. This pattern of variation provided further evidence that the sequences are genuine and not the result of numts. Further investigation and additional samples will be required to determine the potential origins and range of this early diverging *M. antilopinus* lineage.

The three remaining clades are geographically distinct with the intervening areas possibly representative of barriers to dispersal. The earliest divergence event that we

recovered (52,000 years ago) was that separating the two clades currently found in the Northern Territory. The boundary between these two clades does not coincide with known geographic barriers in the area but may represent a past vicariant event followed by a secondary re-colonisation by the NT1 clade from the Kimberley eastward or NT2 clade moving westward, resulting in the two clades coming together. Something similar has been hypothesised for rock wallabies in northern Australia where an identical geographical pattern was seen (Telfer & Eldridge 2010). In the arid conditions during glacial maxima, species distributions would have contracted their distributions to sites where moisture and food resources remained available. Therefore, the escarpments of Arnhem Land and the Kimberley could have acted as major refugia at these times (Telfer & Eldridge 2010). Isolated populations in these refugia could have diverged genetically over time and then when climates again became wetter and food resources more broadly available, *M. antilopinus* would have dispersed and recolonised habitat patches across the landscape. The lack of geographic overlap seen between the NT clades (Figure 5) may be an artefact of a secondary contact zone for two diverged lineages of *M. antilopinus*. Other possible hypotheses to explain the geographic separation are modern barriers, including the highway separating two large national parks, Litchfield and Kakadu (Figure 5) or possible changes in elevation and sandstone outcrops (see Figure 1 from Bowman *et al.* (2010)) affecting dispersal and integration due to changes in habitat. The PCoA analysis (Figure 4) revealed a slight segregation between the two WA samples and the lowest latitude NT sample (ABTC27780) compared to the remaining NT1 samples. This separation possibly coincides with the Kimberley Plateau–Arnhem Land barrier, resulting from the Victoria River Drainage Barrier or Ord Arid Intrusion. More extensive sampling from this area and the surrounding Kimberley region would be required to confirm the degree of differentiation and if this region acts as a biogeographic barrier for *M. antilopinus*.

The remaining mtDNA clade contained samples found only in Queensland. This separation of lineages coincides with the known split in the species distribution (Figure 1) and confirms the lack of dispersal across the Carpentarian barrier. This gap in the species distribution has been attributed to the formation of the Carpentarian plains containing unsuitable habitat for *M. antilopinus*, which has prevented subsequent dispersal and gene flow between QLD and NT populations (Ritchie *et al.* 2008). Despite the presence of a number of well characterised barriers in north eastern Australia, there appears to be no phylogeographic structuring of *M. antilopinus* within this part of its range.

The challenge for historical biogeography is to establish whether there is congruence among different lineages with respect to the timing of divergence of ancestral populations and, if so, whether divergence times coincide with major geological events, such as the formation of the Carpentarian Barrier (Braby 2008). The initial split between the western (NT1 and WA) and eastern clade (NT2 and QLD) at 52, 000 years ago maybe associated with northern Australia becoming wetter about 60, 000-30, 000 years ago (Ward *et al.* 2005), when it is believed the Australian monsoon was at full strength (Johnson *et al.* 1999).This could have resulted in increases in the extent of floodplains, possibly isolating macropod populations. The divergence estimate of 34,000 years for QLD samples from NT2 samples is congruent with the beginning of the last glacial maximum, dramatic drop in sea levels with the associated cooling and increases in aridity across northern Australia (De Deckker *et al.* 1991). This change in climate resulted in Lake Carpentaria spreading to its largest extent before intrusion of the sea, possibly affecting dispersal due to unsuitable habitat surrounding the brackish lake. Since the last glacial cycle, Lake Carpentaria merged with rising sea levels to form the Gulf of Carpentaria and the Arafura Sea, severing connections between northern Australia and

southern New Guinea. As a consequence it is predicted that populations of widespread species became fragmented (Braby 2008).

The effect of biogeographic barriers on population differentiation has been investigated in a number of phylogeographic studies across northern Australia (Lee & Edwards 2008; Fujita *et al.* 2010; Telfer & Eldridge 2010; Toon *et al.* 2010; Melville *et al.* 2011; Smith *et al.* 2011; Potter *et al.* 2012). Many studies have highlighted the importance of the Carpentarian barrier in shaping species distribution and diversification (Cracraft 1986; Cardinal & Christidis 2000; Jennings & Edwards 2005; Lee & Edwards 2008; Kearns *et al.* 2011). Two contradictory phylogeographic patterns across the Carpentarian barrier have been identified for birds; strong differentiation (Lee & Edwards 2008; Toon *et al.* 2010; Kearns *et al.* 2011) and the apparent absence of any impact on species differentiation (Kearns *et al.* 2010; Joseph *et al.* 2011). The results for *M. antilopinus* suggest the Carpentarian barrier has produced strong levels of differentiation in populations on either side pre-dating the Last Glacial Maximum.

During the glacial climatic cycles of the Pleistocene the Carpentarian barrier is likely to have represented a more significant arid barrier than at present (Bowman *et al.* 2010), and has previously been implicated in intra- and inter-species divergences dating from the mid-Pliocene to the late Pleistocene (Jennings & Edwards 2005; Toon *et al.* 2007; Lee & Edwards 2008; Toon *et al.* 2010; Kearns *et al.* 2011). The divergence estimate for the Carpentarian barrier split for *M. antilopinus* is the first estimate for any terrestrial mammal and falls considerable later than the estimates of many bird diversifications (Jennings & Edwards 2005; Toon *et al.* 2010), with the exception of the magpie at 36, 000 years ago (Toon *et al.* 2007). These differences highlight major temporal variation in the way the Carpentarian barrier influenced different taxa.

Conclusion

The contemporary spatial structure of *Macropus antilopinus* gene lineages revealed three clades that provide some clear signal into the possible biogeographic barriers affecting the species genetic history and a fourth, older lineage of unknown origin and range. The contemporary genetic differences observed within the Northern Territory samples (observation of two clades) was unexpected, as there is no known biogeographic barrier in that region of the Northern Territory (Joseph & Omland 2009; Potter *et al.* 2012). The genetic differences that we found between the Queensland and the Northern Territory *M. antilopinus* samples, confirmed the split in geographical distribution of the species. This split coincides with the Carpentarian barrier representing unsuitable habitat for the species. We also found weak genetic distinctions between Northern Territory samples and those from the Kimberley region of Western Australia, possibly due to the Kimberley Plateau-Arnhem Land barrier. These geographical patterns highlight the importance of investigating genetic variation across species' distributions. Although presently widespread and not considered threatened the antilopine wallaroo shows clear genetic distinction that will need to be considered in future management and conservation initiatives.

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Supplementary Material

Table S1. List of *Macropus antilopinus* samples used for phylogeographic analyses showing museum numbers and sample locations.

Sample number	Museum Accession Number	Museum / supplier	Sample type	State	Basic Location	Latitude	Longitude
ABTC27780	ABTC27780	ABTC	Tissue	NT	Gregory NP	-16.8045	130.166
ABTC83375	ABTC83375	ABTC	Tissue	QLD	Mt Surprise	-18.1	144.4
ABTC83671	ABTC83671	ABTC	Tissue	NT	Katherine	-14.8174	131.9504
11878	CM5044	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11880	CM1032	ANWC	Bone	NT	Port Bremer	-11.2	132.25
11883	CM534	ANWC	Bone	NT	Kakadu NP	-12.7583	132.6567
11884	CM552	ANWC	Bone	NT	Kakadu NP	-13.0667	132.45
11885	CM757	ANWC	Bone	QLD	Moreton	-13.1667	142.8
11888	CM3338	ANWC	Bone	NT	Darwin	-12.45	130.8333
11889	CM3402	ANWC	Bone	NT	Adelaide River	-13.2397	131.1065
11890	CM5040	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11891	CM5041	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11892	CM5042	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11894	CM5045	ANWC	Bone	NT	Gimbat Station	-13.5833	132.6
11895	CM7037	ANWC	Bone	NT	Kakadu NP	-12.8667	132.8
11897	CM7120	ANWC	Bone	NT	Kakadu NP	-12.8667	132.8
11900	CM7902	ANWC	Bone	NT	Kakadu NP	-13.4667	132.4833
M37010	M37010	ANWC	Tissue	QLD	Mt Surprise	-18.1	144.4
11902	U0018	MAGNT	Bone	NT	Darwin Area	-12.45	130.83
11903	U2515	MAGNT	Bone	NT	Heathers Lagoon	-12.92	131.23
11904	U2516	MAGNT	Bone	NT	Stapleton Station	-13.08	130.17
11907	U2518	MAGNT	Bone	NT	Stapleton Station	-13.08	130.67
11914	U5769	MAGNT	Bone	NT	Palmerston	-12.51	131.02
11231	M8423	SAM	Bone	NT	Stapleton Creek	-13.18	131.03
11232	M276	SAM	Bone	NT	Mary River	-13.5	132
11234	M278	SAM	Bone	NT	Mary River	-13.5	132
11235	M349	SAM	Bone	QLD	Coen River	-13.67	142.75
11236	M8421	SAM	Bone	NT	Pine Creek	-13.83	131.83
11921	M17271	WAM	Bone	NT	Kakadu NP	-13.25	132.5
11924	M19959	WAM	Bone	WA	Kalumburu	-14.3	126.6333
11929	M21259	WAM	Bone	WA	Mitchell Plateau	-14.75	125.75
Ma1		Euan Ritchie	DNA extract	QLD	Rocky Springs Station	-18.1	144.4
Ma2		Euan Ritchie	DNA extract	QLD	Kendall River Station	-13.75	142.183
Ma3		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma4		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma41		Euan Ritchie	DNA extract	QLD	Georgetown	-18.292	143.55
Ma42		Euan Ritchie	DNA extract	QLD	Croydon	-18.205	142.249

Ma43		Euan Ritchie	DNA extract	QLD	Croydon	-18.205	142.249
Ma5		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma6		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma7		Euan Ritchie	DNA extract	QLD	Merluna Station	-13.0167	142.9833
Ma8		Euan Ritchie	DNA extract	QLD	Merluna Station	-13.0167	142.9833
Ma9		Euan Ritchie	DNA extract	QLD	Chillagoe	-17.154	144.523
Ma10		Euan Ritchie	DNA extract	QLD	Bramwell	-12.1422	142.6227
Ma1002		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma0601		Euan Ritchie	DNA extract	QLD	Rocky Springs Station	-18.1	144.4
Ma0602		Euan Ritchie	DNA extract	OLD	Rocky Springs Station	-18.1	144.4
Ma0603		Euan Ritchie	DNA extract	QLD	Lakeland	-15.862	144.858
Ma0604		Euan Ritchie	DNA extract	QLD	Georgetown	-18.292	143.55
Ma0605		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316

ABTC = Australian Biological Tissue Collection at the South Australian Museum, ANWC = Australian National Wildlife Collection (CSIRO Ecosystem Sciences), MAGNT = Museums and Art Galleries of the Northern Territory, SAM = South Australian Museum. NT = Northern Territory, QLD = Queensland, WA = Western Australia, NSW = New South Wales, SA = South Australia.

Chapter VII: Concluding discussion

Antilopine wallaroo © Australian Wildlife Conservancy
http://www.australianwildlife.org/Wildlife-and-Ecosystems/Wildlife-Profiles/Mammals/Antilopine-Wallaroo.aspx

Chapter VII: Concluding discussion

This thesis contains a portfolio of standalone chapters containing detailed discussions and conclusions of the research presented therein. In this chapter, I first review the aims of the study, and then give an overview of the significance of the findings and their implications to the utilisation of non-invasive sampling for conservation and management. Finally, I outline the limitations of the study and point out some recommendations for future research.

Review of aims

This project aimed to:

- Develop a quick and inexpensive method to identify macropod scat samples to species level.
- Investigate whether molecular surveys, using non-invasive genetic methods, can be used to obtain occurrence data for fitting ecological models.
- Isolate microsatellite loci for the antilopine wallaroo and design multiplex PCR reactions to enable cost-effective population genetic analyses.
- Assess fine-scale population structure and dispersal of the antilopine wallaroo in Cape York using microsatellite data from field collected scats.
- Determine the phylogeography of antilopine wallaroo by analysing historical aspects of the contemporary spatial distributions of gene lineages in relation to known geographical barriers.

Aim 1

Summary and significance

Chapter II demonstrates the possibility of using non-invasive sampling to identify species and obtain accurate location presence points for sympatric species. These accurately distinguished samples can then be used to determine species presence, abundance, distributions, habitat preference as well as in downstream genetic analysis. The main finding is that using the restriction fragment length polymorphism (RFLP) based identification method provides a powerful discriminatory tool for the quick and inexpensive identification of macropod species. This allows indirect non-invasively collected samples from sympatric species to be identified accurately. The newly designed macropod identification method eliminates the need for sequencing, which is costly, time-consuming and requires additional laboratory equipment, as well as visual confirmation. The genetic identification of species method has further applications, including the identification of kangaroo products. For example, the method could be used to monitor species killed as part of the legal commercial harvest and to identify contaminated or substituted meat products (Moore *et al*. 2003; Haider *et al*. 2012).

Beyond determining a quick and inexpensive method to distinguish species, the research presented in this thesis has general implications for the field of conservation genetics. This has included revealing that easily collected non-invasive samples, such as scats, can provide an alternative source of DNA for species occurrence as well as further genetic analysis. Chapter II shows the benefits of utilising non-invasively collected samples in a practical context by conducting both an *in-situ* test followed by assessing the field-based application. In the field, accurate visual identification of macropod species responsible for a scat is extremely difficult. Many species are sympatric and their scats are very similar morphologically and in terms of their internal composition. Errors in visual identification of

non-invasive samples can lead to false positives and false negative species identifications (Janecka *et al*. 2008; Harrington *et al*. 2010). Such errors can lead to biases in ecological model estimates of abundance and distribution, wasted resources and misdirected management and conservation actions.

My research in Chapter II has addressed some of the negative aspects associated with non-invasive sampling. Most importantly addressing the problems associated with accurate identification of non-invasively collected samples and the costs involved in processing the samples. Our macropod identification results using field scats have improved our understanding of the spatial distribution of sympatric macropods in north-eastern Australia. Five of the six species identified from scat samples were found outside their known distributions. Unlike visual surveys where presence/absence points cannot be double-checked at a later date, scat identification can be checked by repeating the extraction and DNA testing, thus eliminating the chance of false-positives.

It is hoped that molecular identification of scats could replace or supplement existing methods for distribution and abundance surveys where accurate species identification is required. The RFLP identification test is fast, cost-effective and highly accurate, where access to a typical molecular biology laboratory is available. With an *in situ* planning phase this test could be applicable to many other species. Initially, the level of sequence variability would need to be investigated to identify diagnostic restriction sites that, with the appropriate endonucleases, produce unique fragment patterns.

Limitations and future directions

To standardise approaches for utilising non-invasive samples, such as scats, it would be useful to investigate several research avenues that were not addressed in this thesis. These include

investigating the impact of a wider range of sampling methods on the recovery of DNA from scats, such as: (i) the influence of time since deposition on the amount of DNA; (ii) the effect of environmental conditions on scat DNA survival; and (iii) investigating the difference in DNA yield from the inside versus the outside of scats.

The designed RFLP identification method relies on species having fixed nucleotide differences to allow specific endonucleases to cleave fragments. Any mutations within the recognition sites would result in the endonucleases not digesting the fragment. This was observed for one of the red kangaroo scat samples containing a mutation removing one of the restriction sites. The identification test was designed to have multiple restriction sites per species, to reduce the risk of misidentification. However, as shown in Chapter II misidentification may still occur. In this instance the error was easily identified due to the unlikelihood of black-striped wallaby being found where the misidentified red kangaroo scat sample was found. The addition of species specific endonucleases or sequencing can be used to confirm questionable samples.

Mutations may also occur elsewhere (not within restriction sites) in the amplified fragment possibly creating new restriction sites not previously considered in the *in situ* tests. This would result in additional fragments being produced. If this was the case, those samples could be easily identified by an extra band being produced in the fragment pattern. These samples may still be distinguishable from the known fragments due to including multiple restriction sites. However, to be sure of the accuracy, the sample can also be sequenced to confirm the species identity. Once the fragment pattern is identified, that specific pattern can be added to the possible outcome of that species.

This macropod identification test was designed using sequence variation of macropods found in the north-eastern region of Australia. Therefore, it does not include all the *Macropus* species or all the possible levels of variation found Australia wide. For the macropod species included in this test, using it in other regions would require extra caution due to the increased likelihood of variation being present in the sequence. To include the additional *Macropus* species, the fragment would need to be sequenced and an *in situ* test conducted. It would need to be determined if using the three endonucleases would result in a distinctive fragment pattern compared to the sympatric species in the area likely to be encountered. If an informative pattern is not produced addition or replacement endonucleases would need to be included.

Aim 2

Summary and significance

Chapter III is a proof of concept study showing the suitability of using indirect genetic surveys of non-invasively collected samples for monitoring macropod occurrence. This study extended the molecular species identification method from Chapter II by using statistical models to investigate the environmental correlates of genetic occurrence data and directly exploring the benefits of indirect genetic versus visual survey methods. Here I provided a field-based analysis of the two survey methods to assess the effectiveness of conducting an indirect genetic survey of macropods. Indirect genetic surveys produce more reliable and less biased occurrence data than visual surveys for highly vagile species, such as macropods, providing a strengthened description of the environmental correlates of occurrence. Furthermore indirect genetic surveys are significantly cheaper to conduct and less time consuming.

Surveying the distribution, habitat use and response to threatening processes of species is critical to the design and monitoring of management and conservation programmes (Clarke *et al*. 2001). Therefore, methods that produce more reliable occurrence data, such as indirect genetic surveys, are particularly important and have many potential benefits and uses for both management and conservation (Ritchie & Bolitho 2008). Indirect genetic surveys are a cost effective method for collecting species occurrence data, which can be directly used in ecological models to evaluate potential drivers of species distributions. The results from these models can then be used to inform the management of species today and in the future (Araujo & Peterson 2012).

By providing more precise occurrence data for considerably less cost, I anticipate that molecular techniques will strengthen previous efforts to model climate change impacts on macropods using species distribution models. I conclude that indirect surveys using molecular

approaches have an important role to play in developing future management practices and guidelines to aid species conservation.

Limitations and future directions

As a proof of concept study, there are many additional applications for indirect genetic surveys not investigated in this study. These can include investigating the number of scats needed to be collected to obtain an accurate representation of macropods at a given site/area and ways to utilise this information in more sophisticated occupancy and abundance models that account for probability of detection. To determine abundance, studies would need to be undertaken to determine the deposition rates for each species. In addition, previous methods for determining the abundance of macropods required an entire site to be cleared of all scats and then scats collected over a period of time (Wiggins & Bowman 2011). This method severely limits the number of sites able to be surveyed and dramatically increase survey costs.

This study specifically looked at occurrence, eliminating the need to clear sites but was still limited by the number of sites surveyed and the number of scats collected. A larger sample size of collected scats for identification and an increase in the number of sites surveyed would provide more presence and absence data, which could further strengthen the ecological-model results. Additional sites should be chosen to better capture the entire range of the macropod species being investigated, to allow for a better representation of the climatic and environmental factors affecting the species distribution. Our results of the climatic and environmental predictors for the four species are specific to those factors affecting the distributions and range dynamics in north-eastern Australia.

The results for the visual survey alone could not be compared directly with the indirect genetic survey as the visual assessment data did not follow linear assumptions and therefore

the models did not converge. To be able to compare the technique directly more complex occupancy models would need to be applied, or a greater number of sites surveyed. However, with the limited survey data it was decided to clearly show that the visual data does not add any extra statistical value, over and above the indirect genetic survey data. Therefore, for species where their occurrence has been patchily surveyed models can be developed with just indirect genetic survey data.

Aim 3

Summary and significance

Chapter IV outlines the isolation of microsatellite loci and the design of multiplex reactions to enable cost effective downstream genetic analysis of antilopine wallaroo scat samples. This study provides a set of microsatellite loci that are informative for population genetic studies of antilopine wallaroo and also possibly the common wallaroo, although probability of identity and heterozygosity was not assessed in this sister species. Twelve of the 15 loci were polymorphic in the antilopine wallaroo samples tested and in combination provide high individual discriminatory power. The chosen microsatellite loci and primers were actually determined from a common wallaroo (*Macropus robustus*) sample so I believe they will also be informative for this species. However, initial testing and possible redesign of the multiplex reactions would be required for the common wallaroo to ensure loci sizes don't overlap.

The loci were chosen to be effective for non-invasive samples, such as the scats identified in chapter II, by minimising the fragment length to increase the likelihood of amplification in possibly degraded samples. An iterative, multi-tube approach was used to reduce error rates and obtain reliable consensus genotypes. Multiplex PCR reactions were designed to decrease the cost of analysis, while allowing for replication to form an accurate consensus genotype from non-invasively collected samples. In conclusion, the genetic variability observed for these loci indicate their potential as suitable population genetic markers in studies of conservation and management of antilopine wallaroo and possibly the common wallaroo and other macropods.

Limitations and future directions

To obtain a DNA extract with a high DNA concentration, museum tissue samples were utilised. One of the major assumptions of utilising museum samples is that sample details and

species identification are accurate. For this study, this was not the case for a number of tissue samples. Of the tissue samples obtained for the RFLP identification test (Chapter II) and additional antilopine wallaroo samples, six samples were found to be misidentified. Unfortunately the inaccuracy was discovered after one of the samples was sent away for next generation sequencing. Therefore, the microsatellite loci were actually derived from a common wallaroo sample, creating a possible ascertainment bias by using microsatellite loci designed from a different species.

Microsatellite loci chosen on the basis of being highly polymorphic in one species will generally tend to harbour shorter repeats in a related species creating an ascertainment bias (Ellegren *et al*. 1995). Generally, most studies select loci based on their high variability resulting in estimates of heterozygosity that are biased upward (Rogers & Jorde 1996). Our loci, although designed in a different species, were not directly chosen based on levels of polymorphism so may therefore represent a more accurate representation of overall heterozygosity even in the sister species antilopine wallaroo. A future study testing these loci on common wallaroo samples would be interesting to see if the levels of heterozygosity are similar in the two species.

Following initial screening of the chosen 17 loci, two were excluded due to consistent amplification failure or interference with other loci. The design of the multiplex reaction resulted in some loci peaks being in a similar size range as others. Although varying the fluorescent dye label allows for these peaks to still be included in the same multiplex reaction, two of the loci were constantly interacting creating indistinguishable pull up peaks. As such one of these loci was dropped from the set of microsatellites to be used in the study. The second locus to be excluded was due to consistent amplification failure in the scat samples. This locus was the one penta-nucleotide included and one of the largest microsatellites in the

set. One of the alleles observed in some of the higher quality samples was approximately 280 bp. This was making the accuracy of calling alleles for the loci difficult due to the likelihood of larger alleles dropping out in poorer quality samples.

Aim 4

Summary and significance

Chapter V examines the population genetics of two populations of antilopine wallaroo using the scat samples extracted for chapter II and the microsatellite loci isolated in chapter IV. The two populations were from the latitudinal extremes of the antilopine wallaroo's Cape York distribution. Genetic studies can be valuable in identifying the impact of anthropogenic processes and so enable better informed conservation management decisions. They also allow management decisions to be made that conserve important evolutionary processes. By utilising non-invasively collected samples I determined the genetic diversity to be low, and found restricted levels of contemporary gene flow resulting in genetic structuring in the antilopine wallaroo populations. The heterozygosity levels seen in these antilopine wallaroo populations are dramatically lower than most other macropodoids studied, including large/wide-ranging species and small/restricted species. Restricted levels of contemporary gene flow were also found despite the presence of continuous habitat in north-east Queensland.

This study shows the potential of utilising both hyper-variable genetic markers, such as microsatellites, and non-invasively collected samples, such as scats, to investigate ecological patterns within wild macropod populations. This study provides baseline data on the genetic diversity, population structure and contemporary levels of gene flow among antilopine wallaroo within continuous habitat in the north-eastern distribution of this species. Unlike other large vagile mammals, the antilopine wallaroo displays low genetic diversity and lack of gene flow even in continuous habitat. This improved knowledge on population genetics can aid in understanding macropod population biology, behaviour and reproduction, directly effecting conservation and management plans for the antilopine wallaroo. It is hoped in the future, more population genetic studies will utilise non-invasive sampling especially for large,

widespread and abundant macropod species rather than just threatened and fragmented species most commonly studied.

Limitations and future directions

Of the 165 antilopine wallaroo scat samples identified in Chapter II, 113 were able to be successfully genotyped for all 15 loci. An overly conservative approach was applied by excluding all samples with only partial genotypes. This was implemented in an effort to minimise the risk of including samples affected by genotypic errors such as null alleles. In order to ensure that no antilopine wallaroo individual was sampled more than once, identical genotypes found at the same site (e.g. within approximately 15 km) were excluded. For other large vagile species this exclusion process may not be effective enough, (due to large home ranges or dispersal ability) to ensure individuals are not resampled and more stringent guidelines may need to be adopted.

Only two populations were sampled in Chapter V from the latitudinal extremes of the antilopine wallaroo's north-eastern distribution. To effectively determine the population structure across the entire Queensland region samples representing the entire range would need to be included. Most importantly this would include samples between the two populations to determine the extent of dispersal and population structuring. Due to time and cost restrictions, scat samples from the remaining antilopine wallaroo distribution across northern Australia were unable to be obtained. Therefore, it is unknown if the population structure and heterozygosity found in Queensland is similar to that found in the Northern Territory or Western Australia.

Advances continue to alter and expand the potential applications of available genetic techniques. Genomics is becoming increasingly employed as the number of species with

sequenced genomes and ‘genome enabled’ species increase (Luikart *et al*. 2003; Kohn *et al*. 2006). The development of next generation sequencing technologies, which enable deep sequencing of samples, has provided a way of obtaining nuclear DNA from degraded samples (Stiller *et al*. 2009). Metagenomic sequencing of samples increases the ability to recover nuclear DNA by providing deep sequencing of all areas of the genome, compared to PCR based approaches. Even though whole-genome sequencing is becoming increasingly more affordable, phylogeography and phylogenetics studies rely on the analysis of hundreds of individuals. Therefore, methods that reduce the genome to a subset of loci should remain more cost-effective for some time to come (McCormack *et al*. 2013).

Aim 5

Summary and significance

Chapter VI investigates the effects of geography on the levels of genetic diversity in antilopine wallaroo. The challenge for historical biogeography is to establish whether there is congruence among different lineages with respect to the timing of divergence of ancestral populations and, if so, whether divergence times coincide with major biogeographic events, such as the formation of the Carpentarian Barrier (Braby 2008). The contemporary spatial structure of antilopine wallaroo gene lineages found three clades that provide some clear signal into the possible biogeographic barriers affecting the species genetic history. The divergence time estimates for the three clades can all be linked to environmental changes occurring across northern Australia during the Late Pleistocene.

The contemporary genetic differences observed within the Northern Territory samples (observation of two clades) was unexpected, as there is no known biogeographic barrier in that region of the Northern Territory (Joseph & Omland 2009; Potter *et al*. 2012). The divergence estimate for these two distinctive genetic groups of antilopine wallaroo was determined to be 52,000 years ago. One hypothesis for this split is due to the increased monsoonal activity in northern Australia resulting in increased flooding and isolating populations.

Genetic differences were found between the Queensland antilopine wallaroos and the Northern Territory samples confirming the split in geographical distribution of the species. This split coincides with the Carpentarian barrier separating Queensland and the Northern Territory. The divergence estimate of 34,000 years ago is congruent with the commencement of the last glacial maximum and the dramatic changes resulting from the change in climate and sea-level. Weak genetic distinctions were also found between Northern Territory samples and those from the Kimberley region of Western Australia possibly representative of the

Kimberley Plateau-Arnhem Land barrier. However, further sampling would be required to confirm the extent of this divergence. Both the Carpentarian and Kimberly Plateau-Arnhem Land barrier have been confirmed in a number of other species across northern Australia (Jennings & Edwards 2005; Braby 2008; Lee & Edwards 2008; Bowman *et al*. 2010; Toon *et al*. 2010; Eldridge *et al*. 2011; Potter *et al*. 2012; Rollins *et al*. 2012).

The resulting phylogeographic patterns observed in the antilopine wallaroo can be associated with known biogeographic barriers and climatic changes in northern Australia. These patterns highlight the importance of investigating genetic variation in species. Although presently widespread and not considered threatened the antilopine wallaroo shows clear genetic distinction that will need to be preserved.

Limitations and future directions

Analysis of a wider range of geographically spaced samples than were examined in Chapter VI would provide a detailed picture of possible biogeographic barriers affecting historical movement of antilopine wallaroo. The areas where increased sampling would have been particularly informative would be from the Kimberly region in Western Australia and the area surrounding the Kimberley Plateau-Arnhem Land Barrier to try and infer the possible involvement of any of the known barriers, e.g. the 'Ord Arid Intrusion' (Bowman *et al*. 2010), 'Bonaparte Gap' (Toon *et al*. 2010), or Victoria River Drainage Barrier (Joseph & Omland 2009).

Antilopine wallaroo DNA was obtained from museum tissue samples, museum bone samples and previous DNA extracts. One of the major assumptions of utilising museum samples is that sample details are accurate. For this study this was not the case, five of the nine

antilopine wallaroo tissue samples and four of the 54 bone samples obtained were different species.

A high DNA extraction success rate of 54 out of 63 was observed for the museum bone samples. The majority of these samples were mummified cartilage from inside the nasal cavity of museum-prepared skulls. These samples would have been protected from the external environment prior to collection and from the ambient museum environment after collection. The lower success rate for the Western Australian antilopine wallaroo (six out of 12) is likely to be due to many of these museum samples being skin samples. Museum skins are likely to have been treated during the museum preservation process, which can negatively affect DNA extraction and amplification success. Of the 50 bone samples that were identified to be antilopine wallaroo, 27 produced high quality sequence for all fragments needed to obtain the required length of ND2, cytb and CR. The remaining samples produced partial fragments for one or more of the regions.

With the advent of the genomic era and next generation sequences technologies, future work on organisms will be able to utilise a vast array of information that would previously have taken years to produce. One major advantage for phylogeographic studies, like that shown for population genetic studies, is the possibility of producing complete mtDNA genomes for all samples. Mitochondrial DNA is a useful popular marker in molecular ecology, population genetics, evolutionary biology, as well as in phylogeographic studies (Avise *et al*. 1987). A high mutation rate, lack of recombination, maternal inheritance and high copy number often makes mtDNA the molecular marker of choice. Until recently, sequencing of mitochondrial genomes was challenging and resource demanding often involving long-range PCR (Gonder *et al*. 2007). As mentioned above the success extraction of DNA from museum samples was quite high but not all samples amplified and produced quality sequences for all

the required overlapping mtDNA fragments. Next generation sequencing technologies are a possible solution for obtaining more data from these degraded samples.

Next generation sequencing employs micro and nanotechnologies to reduce the size of sample components enabling massively simultaneous parallel sequencing reactions to be run. To utilise next generation technologies, extracted DNA is fragmented, repaired and adapters added to form DNA libraries. For well-known model organism, with mitochondrial reference genomes available, capture protocols and subsequent next generation sequencing of enriched DNA templates is a highly effective approach (Mamanova *et al*. 2010). In the absence of closely related reference sequences, this strategy can become problematic but not impossible due to the number of mismatches in the sequence comparison (Hahn *et al*. 2013). A reference sequence from a too distantly related species will only allow the identification of reads mapping to the highly conserved regions of the target species mtDNA. For this reason *de novo* assembly, the reconstruction of genomic sequences without any reference information has become increasingly important. Obtaining complete mtDNA genomes will enable much more detailed studies of evolution, population genetics and phylogeography.

The results presented in Chapter VI are based on the analysis of maternally inherited mtDNA and hence provide no direct information about male antilopine wallaroo across northern Australia. It has been suggested that dispersal of macropodoids is predominately male-biased (Eldridge *et al*. 2010). Therefore, the phylogeographic pattern determined using Y chromosome DNA may produce a different hypothesis regarding possible biogeographic barriers or confirm the barriers shown in Chapter VI. Whether these barriers also affect the other macropod species in the region would also be of interest for the conservation and management of northern Australia's tropical savannahs.

Concluding remarks

The results of this study revealed the applications and benefits of utilising non-invasively collected samples and museum specimens to study the distribution, population genetics and phylogeography of a wide-ranging, large marsupial across northern Australia. I used an indirect genetic survey technique utilising non-invasively collected macropod scats to determine the occurrence of four species in north-eastern Australia while investigating possible environmental variables affecting their distributions. Additionally, I demonstrate the value of using non-invasively collected samples as a source of DNA for genetic analyses of species. With the appropriate design of primers for small target fragments, both mtDNA and nuclear microsatellite loci were able to be amplified from potentially degraded macropod scat and museum specimen DNA to investigate population genetics and phylogeography.

The design of accurate species identification tests for indistinguishable non-invasively collected samples will allow for greater uptake of non-invasive monitoring, both ecologically and genetically for a range of additional species. This is particularly important for elusive or dangerous species where direct observation and sampling is difficult. Given the demonstrated power and flexibility of genetic monitoring using non-invasively collected samples, I believe that applications utilising these samples will expand rapidly to answer key ecological, conservation and evolutionary questions. Developments are also expected in the number and classes of molecular markers used for monitoring, demonstrated by the recent advances in population genomics and entire genome typing (Sunnucks 2000).

Genetic markers are capable of elucidating patterns and population histories, which are not apparent using other methodologies. Furthermore, they provide a means of quantifying the extent of dispersal and the filtering influence of features in the landscape, both contemporary and historic. In this study both autosomal microsatellite markers, as well as three regions of

mitochondrial DNA were used to examine various aspects of the biology of the antilopine wallaroo, *Macropus antilopinus*. Although the antilopine wallaroo is an abundant and widespread species in Australia's monsoonal tropics, the genetic data obtained in this study revealed new insights into dispersal, contemporary structure and the influence of biogeographic barriers. These molecular techniques are capable of providing new information on macropods, influencing the implementation of conservation and management actions.

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Appendices

Appendix I: Macropod phylogeny

This appendix is a macropod phylogeny incorporating the *Macropus* tissue samples I acquired for the identification test. This short study was undertaken to determine if I was able to reconstruct the evolutionary relationships between the species and compare it to previously published marsupial and kangaroo phylogenies. This project was not intended to obtain divergence dates as I have only used mtDNA. Only one evolutionary relationship software program was used to get an initial view of how my samples are related. Therefore, this data and results are to be seen as preliminary. To obtain a more robust phylogeny for my *Macropus* samples, the data would need to be re-analysed and compared using additional analysis techniques and with additional nuclear regions sequenced before any definitive conclusions can be made.

Phylogeny of *Macropus* based on two mtDNA genes

Jessica J. Wadley

Introduction

To be able to design the PCR-RFLP identification test for macropods, I needed to obtain sequence data for a number of mtDNA regions from the species likely to inhabit northeast Queensland. As the samples were available and mtDNA sequences were being generated I also decided to produce a phylogeny of *Macropus*. A number of previous studies have constructed phylogenies for marsupials including macropods using a range of different techniques (Kirsch *et al.* 1995; Burk & Springer 2000; Cardillo *et al.* 2004; Bulazel *et al.* 2007; Meredith *et al.* 2008, 2009; Prideaux & Warburton 2010; Westerman *et al.* 2010; Phillips *et al.* 2013). Although limited in scope (only two mtDNA genes) and sample size for some species I have included species that have not previously been examined, thus extending previous work.

The aim of this study was (1) to sequence mtDNA gene regions from macropods found in Queensland to design an identification test and in doing so, (2) reconstruct a phylogeny based on mtDNA and compare it to previously published trees (Figure 1).

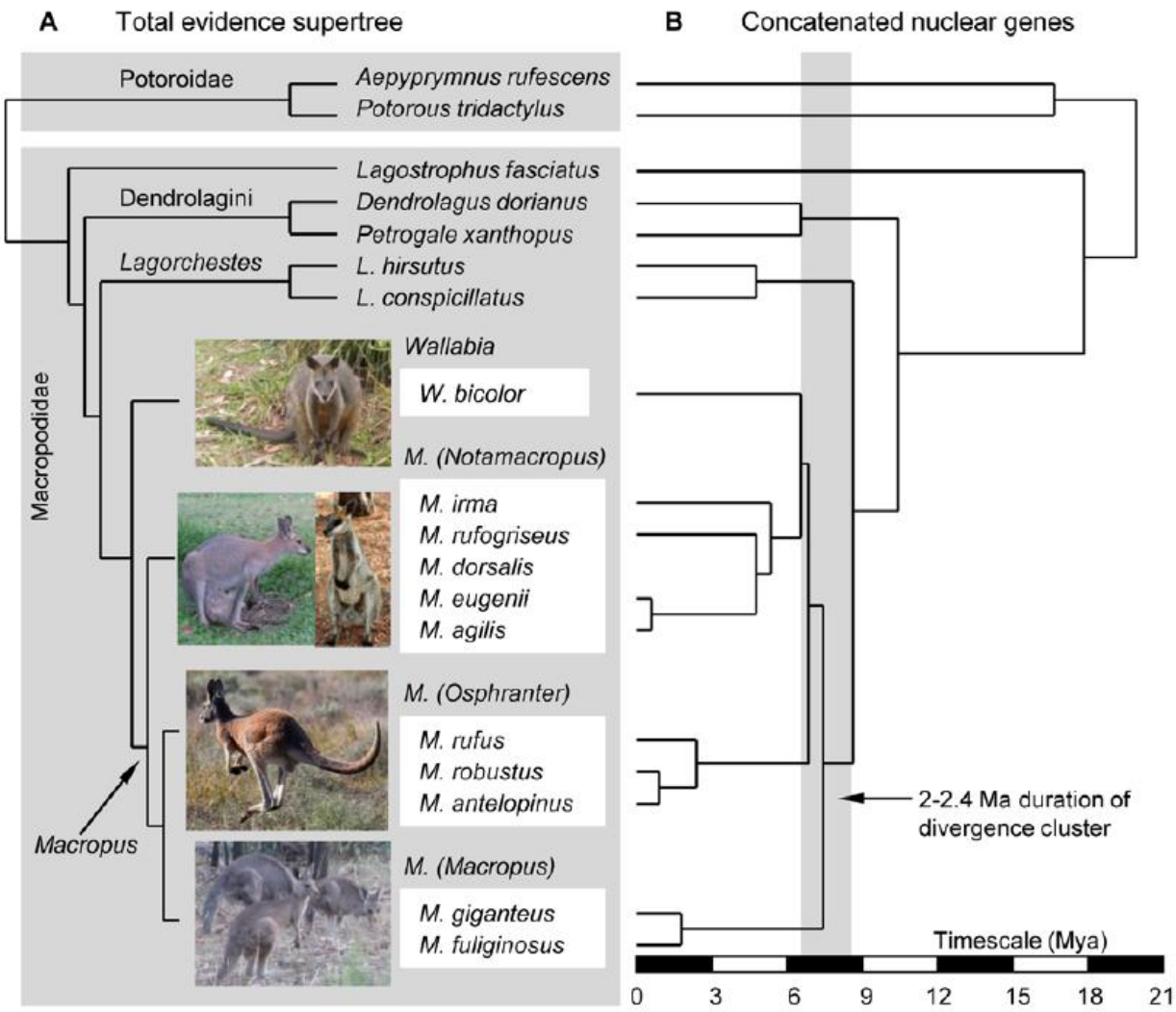

Figure 1. Taken from Phillips *et al.* (2013), showing the current phylogenetic relationship of *Macropus*. (A) The supertree of Cardillo *et al.* (2004) summarizing previous molecular and morphological phylogenies and (B) Meredith *et al.* (2008) based on nuclear genes and including an evolutionary timescale.

Methods

In order to reconstruct a mitochondrial tree for kangaroos, I selected two protein-coding genes, NADH dehydrogenase subunit 2 (ND2) and cytochrome b (cytb). DNA extracts, tissue samples and bone samples were obtained from museum collections and previous studies (Ritchie 2007). The samples included the *Macropus* species, *M. agilis* (agile wallaby), *M. antilopinus* (antilopine wallaroo), *M. dorsalis* (black-striped wallaby), *M. fuliginosus* (western grey kangaroo), *M. giganteus* (eastern grey kangaroo), *M. parryi* (whiptail wallaby), *M. robustus* (common wallaroo), *and M. rufus* (red kangaroo). Genbank sequences for these species were also included where available. Three additional species of *Macropus; M. eugenii* (tammar wallaby), *M. irma* (western brush wallaby) and *M. rufogriseus* (red-necked wallaby) and one sister genus wallaby, *Wallabia bicolor* (swamp wallaby) were also included using sequences available on Genbank. Genbank was also used to locate outgroup samples (*Lagorchestes hirsutus* (rufous hare-wallaby), *Lagorchestes conspicillatus* (spectacled hare-wallaby), *Lagostrophus fasciatus* (banded hare-wallaby), *Petrogale xanthopus* (yellow-footed rock-wallaby), *Dendrolagus dorianus* (Doria's tree-kangaroo), *Potorous tridactylus* (long-nosed potoroo) and *Aepyprymnus rufescens* (rufous rat-kangaroo)).

DNA was extracted from tissue samples using a 'salting out' procedure (Nicholls *et al.* 2000), while DNA was extracted from bone samples using a Qiagen DNeasy blood and tissue kit with the following modification; the amount of ATL, AL and ethanol added were doubled.. Bone extractions were conducted in separate laboratory in a different building to avoid contamination from the tissue DNA and PCR products. All extractions were performed with a negative extraction control to monitor for DNA contamination during the extraction procedures. Bone extracts underwent amplification and sequencing using species identification primers designed in Wadley *et al.* (2013) to confirm species identity.

The two mtDNA fragments, 945 bp of ND2 and 1,104 bp of cytb were concatenated for phylogenetic inference. These fragments have been widely used in macropod phylogenies (Fumagalli *et al.* 1997; Bulazel *et al.* 2007; Phillips *et al.* 2013). Tissue samples and DNA extracts were amplified and sequenced using primers; M1034 and M1035 for ND2 and M441 and M442 for cytb (Table 1). Additional internal sequencing primers were also designed and used; M461 and M440 for ND2; and M462 and M463 for cytb. Tissue sample and DNA extract PCRs were conducted in 25 µl volumes containing 2 µl of DNA, 1x HotMaster PCR Buffer (Eppendorf), 0.2 mM each dNTP, 0.4 µM of each primer and 0.1 U HotMaster *Taq* DNA Polymerase (Eppendorf). Thermocycling was conducted using 2 min enzyme activation at 94 °C followed by 35 cycles of 94 °C for 20 sec, 55 °C for 10 sec and 65 °C for 60 sec, with a final extension step of 65 °C for 10 min. PCR products were purified using AMPure (Agencourt) as per manufacturer's instructions and Sanger sequenced with BigDye v3.1 chemistry (Life Technologies) in 1/16 reactions using the original PCR primers and additional internal sequencing primers. Sequencing reactions were purified using CleanSEQ (Agencourt) as per manufacturer's instructions and run on an ABI 3130XL Genetic Analyzer.

Table 1. Primers used to amplify and sequence modern tissue samples and DNA extracts.

Primer name	Pseudonym	Sequence 5' - 3'	Region	Reference
External				
M1034	Macropus_ND2_F	CCCTTCCCATACTAATGTC	ND2	Designed in this study
M1035	Macropus_ND2_R	GATTTGCGTTCGGATGA	ND2	Designed in this study
M441	Macropus_cytb_Mr1_F	CATTTTAGTATGGACTCTAACCATAACC	cytb	(Bulazel *et al.* 2007)
M442	Macropus_cytb_Mr2_R	AGGGTGTTATACCTTCATTTTTGG	cytb	(Bulazel *et al.* 2007)
Internal				
M461	Macropus_ND2_IntF3	AACAATGCTCGGAGGCTGAG	ND2	Designed in this study
M440	Macropus_ND2_IntR	AGGCTAGGATTTTTCGTAGGTG	ND2	Designed in this study
M462	Macropus_cytb_IntF	GACAAAGCCACCCTCACACGCT	cytb	Designed in this study
M463	Macropus_cytb_IntR	TGTTTCGTGTAGGAATAGGAGGTGGA	cytb	Designed in this study

Bone extractions were amplified and sequenced with a number of short overlapping fragments (Table 2). PCRs performed with DNA extracts from bones samples were conducted in 25 µl volumes containing 3 µl of DNA, 1x High Fidelity PCR Buffer (Invitrogen), 0.25 mM each dNTP, 2 mM $MgSO_4$, 1 mg/ml RSA, 0.4 µM of each primer and 1 U Platinum® *Taq* High Fidelity (Invitogen). Thermocycling was conducted using 2 min enzyme activation at 94 °C followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 30 sec, with a final extension step of 68 °C for 10 min. PCR products were then purified using Millipore MultiScreen PCR_{384}Filter Plates (Millipore) and directly sequenced on an AB 3730*xl* DNA Analyzer (Applied Biosystems) at the Australian Genome Research Facility.

Table 2. Primers used to amplify and sequence museum bone samples.

Primer name	Pseudonym	Sequence 5' - 3'	Region	Reference
A2086	Ma_cytb_Mr1_F	CATTTTAGTATGGACTCTAACCATAACC	cytb	(Bulazel *et al.* 2007)
A2087	Ma_Cytb_mus_intR1	CGTCTCGGCAGATATGGGCA	cytb	Designed in this study
A2088	Ma_Cytb_mus_intF2	ATACCTCAGACACCCTAACAG	cytb	Designed in this study
A2089	Ma_Cytb_mus_intR2	GACAGGAGGTTTGTGATTACGG	cytb	Designed in this study
A2090	Ma_Cytb_mus_intF3	TGTCCTACCATGAGGACAAAT	cytb	Designed in this study
A2091	Ma_Cytb_mus_intR3	GATGGTATAATATGGGTGGAATGG	cytb	Designed in this study
A2092	Ma_Cytb_mus_intF4	TCCGGAATTAACCCCGACTC	cytb	Designed in this study
A2093	Ma_Cytb_mus_intR4	AAGATGGATGCTAGCAGAGC	cytb	Designed in this study
A2094	Ma_Cytb_mus_intF5	CCCTAACAAACTAGGAGGAGT	cytb	Designed in this study
A2095	Ma_Cytb_mus_intR5	GTATGTAGTTTTCAAATAATCCGGC	cytb	Designed in this study
A2096	Ma_ND2_mus_intF1	TCAGCCTCTTCCTAGGCACATCCC	ND2	Designed in this study
A2097	Ma_ND2_mus_intR1	GGAAGGGGGCCAGGCCTAGTTT	ND2	Designed in this study
A2098	Ma_ND2_mus_intF2	ACAGCCTCAGTCCTAATAACCCTAGCA	ND2	Designed in this study
A2099	Ma_ND2_mus_intR2	TCCAGCCTATATGGGCGATGGAA	ND2	Designed in this study
A2100	Ma_ND2_mus_intF3	TGCTCGGAGGCTGAGGAGGC	ND2	Designed in this study
A2101	Ma_ND2_mus_intR3	TGGTATGAAGCCGGTTAGAGGAGGA	ND2	Designed in this study
A2102	Ma_ND2_mus_intF4	CCTCCTCACACTTCTATCTCTAGGCGG	ND2	Designed in this study
A2103	Ma_ND2_mus_intR4	GGGGTGTTAGTGGGAGTAGGAGGG	ND2	Designed in this study

Sequence chromatograms were edited and individual sample contigs assembled using Sequencher 4.7 (Gene Codes Corporation) and sequences aligned using Geneious Pro 5.6.4 (Biomatters). Genbank sequences were aligned and concatenated to create species sequences (Table 3). The kangaroo phylogeny was inferred from the concatenated mitochondrial regions. PARTITIONFINDER version 1.1.0 (Lanfear *et al.* 2012) was run to determine the appropriate models of sequence evolution for the phylogenetic analysis. Reconstruction of a phylogenetic tree was based on Bayesian analysis and employed by the software MRBAYES version 3.2 (Ronquist *et al.* 2012). Four independent runs of Markov chain Monte Carlo analysis were performed with 2,500,000 cycles sampling every 250 cycles. Four chains (1 cold and 3 heated) were used with a temperature of 0.1. The burnin was set to 25 % and branch length information was saved on the sampled trees. Summary statistics were produced for all sampled trees and a consensus tree produced containing the branch lengths and interior nodes labelled with support values. The consensus tree was then visualised using FIGTREE version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 3. Genbank sequences (15) used in the phylogeny, showing species and the accession numbers.

Species	Genbank accession numbers	
	ND2	cytb
Macropus		
M. agilis	AF425981	EF368029
M. eugenii	JN003390	EF368038
M. fuliginosus	JN003399	JN003400
M. irma	JN967006	JN967002
M. robustus	Y10524	Y10524
M. rufogriseus	JN003393	EF368027
M. rufus	JN967007	JN967003
Outgroups		
Aepyprymnus rufescens	JN003377	EU086684
Dendrolagus dorianus	JN003379	JN003380
Lagorchestes conspicillatus	JN003384	JN003385
Lagorchestes hirsutus	NC008136	NC008136
Lagostrophus fasciatus	NC008447	NC008447
Petrogale xanthopus	JN003382	EF368032
Potorous tridactylus	AJ639873	AJ639873
Wallabia bicolor	JN003388	EF368031

Results and Discussion

A total of 86 samples were included in the analysis, 15 concatenated Genbank sequences and 71 museum or tissue samples from this study (Table 3 and 4). The samples covered 11 of the 13 extant *Macropus* species (only missing *M. parma* and *M. bernardus*) and a number of outgroup species. The resulting tree (Figure 2) shows the majority of species, including outgroups, matching the evolutionary relationships determined by Phillips *et al.* (2013). The additional species *M. parryi* not investigated by Phillips *et al.* (2013) fell within the Notamacropus grouping containing *M. rufogriseus*, *M. dorsalis*, *M. eugenii*, and *M. agilis* supporting the placement by Meredith *et al.* (2008). The phylogenetic position of *M. irma* had good support and matched the mitochondrial placement within Osphranter (*M. robustus* and *M. antilopinus*) of Phillips *et al.* (2013) rather than with Notamacropus as seen in Cardillo *et al.* (2004) and Meredith *et al.* (2008). Phillips *et al.* (2013) argued that the position of *M. irma* in the mtDNA tree (which conflicts with nuclear gene trees and morphology) is due to ancient introgression from an ancestral wallaroo. *Macropus giganteus* and *M. fuliginosus* form a clade and matched previous results. However, the phylogenetic position of *M. rufus* contradicts previous molecular and morphological studies, did not have good support and so its placement is suspect. Previous studies have grouped *M. rufus* within Osphranter with reasonable support (Phillips *et al.* 2013). My results add to and in general support the previous knowledge of *Macropus* phylogeny. Additional samples from the majority of species and more in-depth sequencing will be needed to fully deduce the relationships of macropod species.

Table 4**.** Museum and tissue samples for seven *Macropus* species (71) used in the phylogeny, showing museum and locality information. Basic location is to the nearest town or major landmark. Some latitude and longitude data are estimated from locality descriptions.

Sample number	Museum accession no.	Museum / Supplier	Sample type	State	Basic location	Latitude	Longitude
M. agilis							
ABTC83377	ABTC83377	ABTC	Tissue	QLD	Georgetown	-18.2898	143.5477
ABTC83751	ABTC83751	ABTC	Tissue	QLD	Ingham	-18.6511	146.1546
ABTC93299	ABTC93299	ABTC	Tissue	QLD	Katherine	-14.4650	132.2643
M. antilopinus							
ABTC27780	ABTC27780	ABTC	Tissue	NT	Gregory NP	-16.8045	130.166
ABTC83375	ABTC83375	ABTC	Tissue	QLD	Mt Surprise	-18.1	144.4
ABTC83671	ABTC83671	ABTC	Tissue	NT	Katherine	-14.8174	131.9504
11878	CM5044	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11879	M05031	ANWC	Bone	NT	Gimbat Station	-13.5833	132.6
11880	CM1032	ANWC	Bone	NT	Port Bremer	-11.2	132.25
11883	CM534	ANWC	Bone	NT	Kakadu NP	-12.7583	132.6567
11884	CM552	ANWC	Bone	NT	Kakadu NP	-13.0667	132.45
11885	CM757	ANWC	Bone	QLD	Moreton	-13.1667	142.8
11888	CM3338	ANWC	Bone	NT	Darwin	-12.45	130.8333
11889	CM3402	ANWC	Bone	NT	Adelaide River	-13.2397	131.1065
11890	CM5040	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11891	CM5041	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11892	CM5042	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11893	M05043	ANWC	Bone	NT	Douglas Station	-13.5833	131.4
11894	CM5045	ANWC	Bone	NT	Gimbat Station	-13.5833	132.6
11895	CM7037	ANWC	Bone	NT	Kakadu NP	-12.8667	132.8
11897	CM7120	ANWC	Bone	NT	Kakadu NP	-12.8667	132.8
11900	CM7902	ANWC	Bone	NT	Kakadu NP	-13.4667	132.4833
M37010	M37010	ANWC	Tissue	QLD	Mt Surprise	-18.1	144.4
11902	U0018	MAGNT	Bone	NT	Darwin Area	-12.45	130.83
11903	U2515	MAGNT	Bone	NT	Heathers Lagoon	-12.92	131.23
11904	U2516	MAGNT	Bone	NT	Stapleton Station	-13.08	130.17
11907	U2518	MAGNT	Bone	NT	Stapleton Station	-13.08	130.67
11914	U5769	MAGNT	Bone	NT	Palmerston	-12.51	131.02
11231	M8423	SAM	Bone	NT	Stapleton Creek	-13.18	131.03
11232	M276	SAM	Bone	NT	Mary River	-13.5	132
11234	M278	SAM	Bone	NT	Mary River	-13.5	132
11235	M349	SAM	Bone	QLD	Coen River	-13.67	142.75
11236	M8421	SAM	Bone	NT	Pine Creek	-13.83	131.83
11921	M17271	WAM	Bone	NT	Kakadu NP	-13.25	132.5
11924	M19959	WAM	Bone	WA	Kalumburu	-14.3	126.6333
11929	M21259	WAM	Bone	WA	Mitchell Plateau	-14.75	125.75

Ma1		Euan Ritchie	DNA extract	QLD	Rocky Springs Station	-18.1	144.4
Ma2		Euan Ritchie	DNA extract	QLD	Kendall River Station	-13.75	142.183
Ma3		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma4		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma41		Euan Ritchie	DNA extract	QLD	Georgetown	-18.292	143.55
Ma42		Euan Ritchie	DNA extract	QLD	Croydon	-18.205	142.249
Ma43		Euan Ritchie	DNA extract	QLD	Croydon	-18.205	142.249
Ma5		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma6		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma7		Euan Ritchie	DNA extract	QLD	Merluna Station	-13.0167	142.9833
Ma8		Euan Ritchie	DNA extract	QLD	Merluna Station	-13.0167	142.9833
Ma9		Euan Ritchie	DNA extract	QLD	Chillagoe	-17.154	144.523
Ma10		Euan Ritchie	DNA extract	QLD	Bramwell	-12.1422	142.6227
Ma1002		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
Ma0601		Euan Ritchie	DNA extract	QLD	Rocky Springs Station	-18.1	144.4
Ma0602		Euan Ritchie	DNA extract	OLD	Rocky Springs Station	-18.1	144.4
Ma0603		Euan Ritchie	DNA extract	QLD	Lakeland	-15.862	144.858
Ma0604		Euan Ritchie	DNA extract	QLD	Georgetown	-18.292	143.55
Ma0605		Euan Ritchie	DNA extract	QLD	Mt Surprise	-18.148	144.316
M. dorsalis							
ABTC83374	ABTC83374	ABTC	Tissue	QLD	Mt Surprise	-18.1028	144.6087
ABTC83682	ABTC83682	ABTC	Tissue	QLD	Charters Towers	-20.0767	146.2635
M. fuliginosus							
ABTC93725	ABTC93725	ABTC	Tissue	SA	Coulta	-34.4429	135.4563
M. giganteus							
ABTC83581	ABTC83581	ABTC	Tissue	QLD	Prairie	-20.8714	144.6
ABTC83673	ABTC83673	ABTC	Tissue	QLD	Townsville	-18.4061	145.1817
ABTC83852	ABTC83852	ABTC	Tissue	QLD	Cunnamulla	-28.0679	145.6836
M. parryi							
ABTC83438	ABTC83438	ABTC	Tissue	QLD	Mt Fox, Ingham	-18.8228	145.8430
M. robustus							
ABTC83309	ABTC83309	ABTC	Tissue	NSW	Coonabarabran	-31.2739	149.2774
ABTC83370	ABTC83370	ABTC	Tissue	QLD	Bluewater Springs	-19.5195	145.7411
ABTC83379	ABTC83379	ABTC	Tissue	QLD	Mt Surprise	-18.1467	144.3192
ABTC83582	ABTC83582	ABTC	Tissue	QLD	Prairie	-20.8714	144.6
ABTC83661	ABTC83661	ABTC	Tissue	NT	Katherine	-14.8619	131.9311
ABTC83672	ABTC83672	ABTC	Tissue	NT	Katherine	-14.9972	131.8212
ABTC70463	ABTC70463	ABTC	Tissue	SA	Telowie	-33.0543	138.0683
M37011	M37011	ANWC	Tissue	QLD	Mt Surprise	-18.1	144.4833
M37129	M37129	ANWC	Tissue	QLD	Mt Surprise	-18.1	144.4667

ABTC = Australian Biological Tissue Collection at the South Australian Museum, ANWC = Australian National Wildlife Collection (CSIRO Ecosystem Sciences), MAGNT = Museums and Art Galleries of the Northern Territory, SAM = South Australian Museum. NT = Northern Territory, QLD = Queensland, WA = Western Australia, NSW = New South Wales, SA = South Australia.

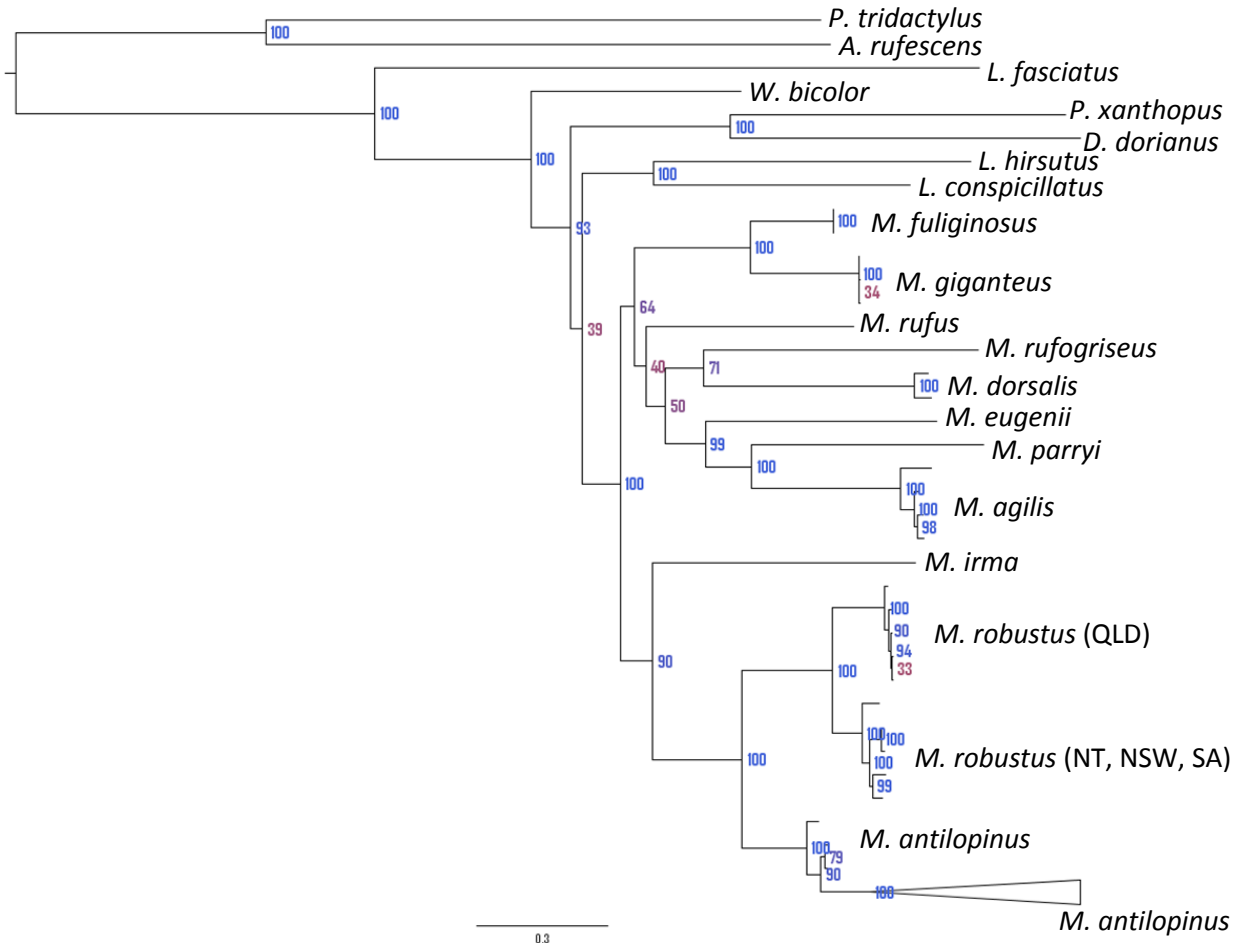

Figure 2. MrBayes tree of evolutionary relationships for concatenated ND2 and cytb mtDNA sequences from 12 species of *Macropus* and eight outgroups. Posterior probabilities for each node are shwon ranging from 1 (red) to 100 (blue). The collapsed branch at the bottom represents the 49 *Macropus antilopinus* samples analysed in chapter VI but without the control region fragment. The early divergent sample from chapter VI (11236) has grouped with two additional *M.antilopinus* samples not included in chapter VI still falling basal to the remaining *M.antilopinus* samples.

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Appendix II: The importance of knowing your roo poo

This appendix is a short communication article based on Chapter II, the development of a PCR-RFLP species identification test designed for macropods. It has been published in the University of Adelaide's e-Science magazine issue 7, October 2013 – Research Bytes, and is available at http://escience.realviewdigital.com/?iid=82481#folio=16.

e-Science is a free magazine, produced by the Faculty of Sciences at the University of Adelaide, and offers interactive feature articles written by researches and complimented by resources specifically designed for teachers.

Wadley, J.J, Austin, J.J. & Fordham, D.A. (2013) The importance of knowing your roo poo. *e-science Magazine, v. 7(October), pp. 16-16*

Appendix III: Did post-glacial changes in sea-level initiate the evolutionary divergence of a Tasmanian ‘endemic’ raptor from its mainland relative?

This appendix contains the publication of research investigating the evolutionary divergence of the Tasmanian wedge-tailed eagle and the possible involvement of changing sea levels. This publication consists of the research completed during my honours degree, incorporated with a number of other studies conducted on wedge-tailed eagles. This article has been published by the *Proceedings of the Royal Society B: Biological Sciences*.

Burridge, C.P., Brown, W.E., Wadley, J., Nankervis, D.L., Olivier, L., Gardner, M.G., Hull, C., Barbour, R. & Austin, J.J. (2013) Did postglacial sea-level changes initiate the evolutionary divergence of a Tasmanian endemic raptor from its mainland relative?.
Proceedings of the Royal Society B: Biological Sciences, v. 280(1773), pp. 1-10