Return to Country: genomics and the repatriation of ancient Aboriginal Australians

A THESIS SUBMITTED TO
SCHOOL OF ENVIRONMENT & SCIENCE
SCIENCE, ENVIRONMENT, ENGINEERING & TECHNOLOGY
GRIFFITH UNIVERSITY
IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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B. Sc (Hons)

Date: 30 April 2018
Abstract

Since European settlement of Australia in 1788, Aboriginal Australian remains have been deposited in museums worldwide. For the past fifty years Aboriginal Australians have campaigned for their 'return to Country'. However, for many remains there are no details of their geographic origins, tribal affiliations or language groups, and as a result they cannot be repatriated.

This research, undertaken in collaboration with Aboriginal Australian Traditional Owners and communities across Australia, tests whether it is possible to determine the origins of ancient individuals using DNA-based methods. Thirty mitogenomes and 10 nuclear genomes from ancient pre-European Aboriginal Australians of known provenance (up to 1,600 yr BP) were recovered and used as proxies for unprovenanced remains. These ancient genomic sequences were compared against assembled reference datasets of contemporary Aboriginal Australian mitochondrial (n=112) and nuclear genomes (n=100).

A number of analytical methods were used to test this potential tool for repatriation including mitochondrial phylogenetics, and nuclear PCA, f3- and f4 outgroup statistics, and chromosome painting symmetry statistics. These analyses showed substantial population structure across Australia. While a distinct east versus west population divide had been observed previously in contemporary Aboriginal Australians, the mitochondrial and nuclear analyses reported here show that this population structure occurs even at a regional level.

Mitochondrial phylogenetetic analyses revealed the majority of mitochondrial haplotypes observed were region-specific, with novel haplotypes being identified in both contemporary and ancient Aboriginal Australians. However, there were also haplotypes which were widespread being observed in a number of Australian states, in addition to rare haplotypes for which there were no contemporary matches. For these ancient remains it was not possible to determine their origin using mitochondrial DNA alone.

The origin of 58.1% of ancient Aboriginal Australians included in this research could be successfully determined using mitochondrial DNA. However, this success was
weakened by results obtained for two ancient individuals, with their respective contemporary matches living on the opposite side of Cape York Peninsula in northern Australia. This is problematic as the return of ancestral remains to an incorrect Country is a major concern of many Aboriginal Australian communities. Therefore, given these erroneous results, only detected because their provenance had previously been established, the reliability of using mitochondrial DNA for repatriation is significantly undermined and must be questioned.

The most accurate results obtained was using nuclear DNA, working in 100% of cases and to precise geographic locations. These results were supported by a number of different analytical methods, each of which independently showed both population structure and local continuity between both the ancient and contemporary populations in each geographic location. However, when combined, these analyses provide strong evidence that nuclear DNA, as a tool for repatriation, is very effective and if applied to unprovenanced ancestral remains will greatly assist with their repatriation.

It is important to note that there are obstacles that need to be overcome before this proposed repatriation method can be put into practice. The first is the cost of completing this type of work. Ancient DNA research is expensive, especially if nuclear DNA is the target of interest, and many museums worldwide will find funding for this sort of research well outside their reach. More significantly though, this DNA–based repatriation tool poses a catch 22 in that the work cannot be undertaken without permission from the appropriate Aboriginal Australian communities, and until the provenance of remains is determined these communities cannot be identified.

A proposed way forward would be national level discussions between the Australian Government, Aboriginal Australians, and museums. These discussions would allow all parties to decide how best to move forward and how the tools presented in this research should be utilised. But ultimately, this is a decision only Aboriginal Australians can make.

This research provides the means to move forward in the repatriation debate, a debate that has caused many Aboriginal Australians considerable sadness and frustration for decades. If
agreement can be reached regarding how it should be implemented, it has the potential to assist in bringing closure to a painful chapter of history for many Aboriginal Australians. While it cannot, and will not correct the mistakes of the past, it may provide healing through the eventual return to Country of many lost ancestors.
Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed) Joanne Louise Wright
Aboriginal Australian & Torres Strait Islander cultural sensitivity warning

Aboriginal Australian and Torres Strait Islander readers are advised that this thesis contains images and discussion of deceased persons, and topics discussed herein may be distressing.

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List of terms and abbreviations

A  adenine
aDNA  ancient DNA
bp  base pair
C  cytosine
G  guanine
HGDP  human genome diversity project
HVS I  hypervariable segment I
HVS II  hypervariable segment II
km  kilometres
KYA  thousand years ago
LGM  Last Glacial Maximum
MDS  multidimensional scaling
ML  maximum likelihood
MRCA  most recent common ancestor
ng  nanogram
np  nucleotide position
PC  principal component
PCA  principal component analysis
PCR  polymerase chain reaction
rCRS  Revised Cambridge Reference Sequence
SNP  single nucleotide polymorphism
T  thymine
TO  Traditional Owner
U  uracil
µL  microlitre
Y-CHR  Y-chromosome
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This thesis is dedicated to the memory of Traditional Owner and Aboriginal Elder of the Thanynakwith People of Weipa, Tapij Wales. Tapij taught me so much about Aboriginal Australian culture, his Country and myself.

You will be sadly missed my friend, and I’m sorry you did not get to see the end result of the research we started together.
Acknowledgements

This body of work would not have been possible without the assistance of many people. It has been a valuable lesson about the synergies involved in large international collaborations. Thanks firstly go to my supervisors, Prof. David Lambert, Dr. Michael Westaway & Dr. Sankar Subramanian. Thank you for allowing me to be involved in such exciting research. Dave, it’s been a difficult journey. Thank you for continuing to offer your support & assistance through to the very end. Our walk to the South Pole is almost over.

To all of the aDNA team who offered much needed support & technical assistance, thank you! To my aDNA lab supervisor Dr Tim Heupink, thanks for helping transform me from a modern DNA girl to an aDNA girl! To my aDNA partner Dr Sally Wasef, thank you for sharing the trials & tribulations of this PhD journey with me. To Dr Richard O’Rorke, thank you for teaching me that chocolate from New Zealand is far superior to that of Australia, a valuable lesson indeed. To Dr Anne Kemp, thank you for our friendship!

I am particularly indebted to the Aboriginal Traditional Owners & communities I had the opportunity to work with, particularly Tapij Wales of the Thaynakwith People of Weipa (sadly deceased before this research was completed). Tapij, it was the highlight of my PhD getting to know you.

Thanks to the Griffith School of Environment & Science, the Environmental Futures Research Institute (especially Dian Riseley – you were a much needed lifeline!) & ARCHE for my PhD scholarship & support. To Dr Eliza Howard & Dr Jim Smart, thank you for always having an open door for me & for all of the excellent advice you’ve given me over the years. I am completely indebted to you both.

Finally, I would like to thank my family, friends, but most of all my partner Lachlan, for their never-ending support, understanding & doses of tough love. Lach, thank you for supporting me through this sometimes painful journey. I’m sorry it was such a rollercoaster ride.
Acknowledgement of papers included in this thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research ("Criteria for Authorship"), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

- Conception and design of the research project.
- Analysis and interpretation of research data.
- Drafting or making significant parts of the creative or scholarly work or critically revising it so as to contribute significantly to the final output.

Section 9.3 of the Griffith University Code ("Responsibilities of Researchers"), in accordance with Section 5 of the Australian Code, states that researchers are expected to:

- Offer authorship to all people, including research trainees, who meet the criteria for authorship listed above, but only those people.
- Accept or decline offers of authorship promptly in writing.
- Include in the list of authors only those who have accepted authorship.
- Appoint one author to be the executive author to record authorship and manage correspondence about the work with the publisher and other interested parties.
- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge.
- Obtain written consent to name individuals.

Included in this thesis are two published papers which are co-authored with other researchers, the respective sections carry the titles of the publications. My contribution to each co-authored paper is outlined at the front of the relevant section. The bibliographic details for these published papers:
**Published Paper 1:**


**Published Paper 2:**


Appropriate acknowledgement of those who contributed to the research but did not qualify as authors are included in each paper.

(Signed) __________________________________________ (Date) 30 April 2018

Joanne Louise Wright

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Supervisor: Professor David M. Lambert
Financial support and the contributions of others

Financial and research support

Financial support for this research was provided by the Australian Government and Griffith University in the form of a PhD scholarship and Living Allowance. Additional funding was provided by the Australian Research Council in the form of grants provided to Professor David Lambert and Dr Michael Westaway, the details of these are as follows:

- Discovery Grant - DP140101405
- Discovery Grant - DP110102635
- Linkage Grant - LP120200144
- Linkage Grant – LP140100387

This research was supported by use of the NeCTAR Research Cloud and by QCIF (http://www.qcif.edu.au). The NeCTAR Research Cloud is a collaborative Australian research platform supported by the National Collaborative Research Infrastructure Strategy.

Contributions of others

This PhD research was a small part of a much larger project which involved a multidisciplinary collaborative team, both from Griffith University and internationally. My contributions to published papers, are outlined on pages 53 and 54 of this thesis.

For my analyses, some of the sequences included were the result of lab work and sequencing completed by Dr Tim Heupink and Dr Sally Wasef. I was assisted with some initial data processing by Dr Tim Heupink, Dr Sally Wasef, Dr Sankar Subramanian and Umayal Ramasamy, all other work is my own or as stated in the accompanying publications.
Map of Australia

For readers who are not familiar with the geography of Australia, the following map has been included showing the major states and territories discussed within this thesis. Common abbreviations for each of these states and territories have been included in parentheses. Additional locations commonly known as Cape York Peninsula, Arnhem Land and the Kimberley have also been shown for clarity.

![Map of Australia](image)

Figure 1 | Map of Australia

**Please note:** for many Aboriginal Australian language sub-groups there are several different variations in the spelling of the name. For each, one was chosen and used throughout this thesis.
Chapter 1: Introduction

1.1 Background

It is beyond the scope of this thesis to discuss in detail the impacts European colonisation of Australia has had on Aboriginal Australians. However, as it is inextricably linked to Aboriginal Australian’s motivations for the repatriation and reburial of institutionalised ancestral remains, this research would be incomplete without reviewing the circumstances that brought Europeans to Australia and the subsequent effects on the Aboriginal Australian population.

1.1.1 Initial colonisation

Aboriginal Australians represent one of the oldest continuous cultures outside of Africa (Hudjashov, et al. 2007; Rasmussen, et al. 2011; Malaspinas, et al. 2016; Nagle, et al. 2017). Much of what we know about the prehistory of these First Peoples comes from archaeological, anthropological, linguistic, and more recently, genetic evidence.

Human settlement of Australia occurred soon after the migration of anatomically modern humans out of Africa, some 60-80 KYA (Roberts, et al. 1994; Thorne, et al. 1999; O'Connell and Allen 2015). Based on the dating of a number of archaeological sites across the Australian continent, initial settlement is thought to have occurred between 40 and 60 KYA (Bowler, et al. 1970; O'Connor 1995; Turney, et al. 2001; Oppenheimer 2009; Clarkson, et al. 2015; O’Connell and Allen 2015) The oldest of these sites, located in Arnhem Land in the north of the Northern Territory, the Madjedbebe (formally known as Malakunanja II) and Nauwalabila rockshelters, have previously been dated using a combination of radiocarbon and luminescence methods to between 53 and 60 KYA (Roberts, et al. 1994; Clarkson, et al. 2015). Though a recent study of Madjedbebe rockshelter using the same methods suggests it was settled between 53.6 and 65KYA (Clarkson, et al. 2017).

For much of the time since its occupation Australia and New Guinea were part of a single enlarged landmass called Sahul (Figure 2). Sea levels were considerably lower than present due to the Pleistocene ice age, with expanses of the continental shelf being exposed, creating land bridges which enabled population migrations (Kohen 1995). For
the same reason, many of the islands of western Indonesia formed a single landmass with mainland Asia, called Sunda. Although this made the passage from Asia to Australia arguably easier for the ancestors of contemporary Aboriginal Australians, Sunda and Sahul never shared a land bridge and remained separated by sea (Birdsell 1977).

Wallacea, encompassing the islands west of Wallace’s Line (i.e. Sulawesi, Flores and Timor), remained separated from Sunda and Sahul with thirty kilometres of open ocean.
separating Sunda and Wallacea, and at least ninety kilometres between Wallacea and Sahul (O'Connell and Allen 2007). In order to reach Australia 40,000 to 70,000 years ago, a minimum of eight to ten sea crossings, or a single crossing of eighty kilometres of open sea was required (Birdsell 1977). While the exact route the founding population likely followed between Asia and Australia has been extensively debated, what is accepted unequivocally is that in order for humans to arrive in Australia both navigation skill and marine technology were required (Macaulay, et al. 2005; O'Connell and Allen 2007; Allen 2008; Balme, et al. 2009).

There has been considerable debate regarding the routes of dispersal taken by the First People after arrival in Australia. Some have proposed hypotheses of rapid dispersal across the continent (Birdsell 1957), while others have proposed migration along water sources such as rivers (Horton 1981; Tindale 1981; Bird, et al. 2016), or along the coast (Bowdler 1977). At the time of arrival, sea levels along the Australian coast were 60 to 80 metres lower than present (O'Connell and Allen 2015)(see Fig. 2). If this route had been taken, migration would have occurred along what is now the continental shelf, and all evidence has long been submerged. The scarcity of Pleistocene dated archaeological sites continues to hinder a number of scientific debates in Australia, from the time and place of arrival of the First People, to megafaunal hunting.

1.1.2 Early contact

For much of the time that Aboriginal Australians have inhabited Australia, the majority of the continent remained isolated from outside populations. The exception was populations living in the northeast where the land bridge between New Guinea and Australia crossed the Arafura Sea, the Gulf of Carpentaria and the Torres Strait. This land bridge was submerged approximately 8 KYA when global warming led to ice sheets melting, increasing sea levels by approximately 100 metres over a period of 5,000 years (Mulvaney and Kamminga 1999). But despite this, even after the land bridge was lost, the northern coastline of Australia continued to be visited by different people for thousands of years (Macknight 1972, 1986; McIntosh 1997; Morwood and Hobbs 1997; Mulvaney and Kamminga 1999).
There has been a long history of contact among Papuan peoples, Torres Strait Islanders and the Aboriginal Australian’s of Cape York Peninsula. The islands of the Torres Strait were settled by seafaring Melanesian cultures over 2500 years ago, and cultural exchange and trade continued via this route (David, et al. 2004). Aboriginal Australians adopted the outrigger dugout canoe, marine technology from New Guinea, and Papuans began to use spears, spear throwers and ochre. Likewise, cultural and trading links across the Torres Strait created a demand for Aboriginal Australian shell ornaments, which were exchanged for various items including ceremonial masks and drums (Mulvaney and Kamminga 1999).

For the Aboriginal Australians of Arnhem Land in the Northern Territory and the Kimberley region of Western Australia, the first outside people they encountered were Macassan sea traders who fished trepang or bèche-de-mer (edible sea cucumbers) in the region probably from the mid 16th century until the 1900s (Macknight 1986; McIntosh 1997; Morwood and Hobbs 1997). The Macassans journeyed from the port town of Makassar in southern Sulawesi (present day Ujung Pandang) each December, returning with the southeast trade winds in March or April the following year (Macknight 1986; Chaloupka 1996). The Macassans often camped on beaches, processed their harvests, and exchanged canoes, sails, hooks, fishing line, beads and metals in order to maintain good relationships with the local Aboriginal Australians, while being granted permission to form relationships with Aboriginal women (Macknight 1986).

From the beginning of the 16th century European merchants and explorers began navigating the oceans surrounding Australia (Lemoine 1807; Heeres and Genootschap 1899; Feeken and Feeken 1970; Henderson 1999). While there is evidence of the Portuguese and Spanish navigating the Torres Strait and New Guinea, the first European explorer known to have sighted the Australian continent was Dutch East India Company captain, Willem Janszoon, who charted the shoreline in 1606. Janszoon arrived in the Gulf of Carpentaria looking to establish new trading ports and collect precious commodities, but found nothing of particular value (Henderson 1999). Janszoon and his crew stepped ashore on western Cape York Peninsula, at the mouth of the Pennefather River near Weipa, where a number of his crew were killed in conflict.
with the local Aboriginal Australian inhabitants (Heeres and Genootschap 1899; Mutch 1942). Further exploration by the Dutch also resulted in violent conflict with Aboriginal Australians in the Gulf of Carpentaria in 1623. The continent was named Nieuw Holland (New Holland) by Dutch seafarer Abel Tasman in 1644, though the Dutch never settled in Australia (Feeken and Feeken 1970).

1.1.3 British arrival and initial settlement

The British first arrived to Australian shores in 1688 when William Dampier travelled to the Kimberley Coast, off Western Australia. Dampier recorded his first impressions of the Aboriginal Australians he had encountered when he made landfall:

“The inhabitants are the most miserable wretches in the universe, having no houses, or covering but the heavens; nor garments, except for a piece of bark from a tree, tied like a girdle around the waist; no sheep, poultry, or fruits, but feed upon a few fish, cockles, mussels and periwinkles; without religion or government, but cohabit promiscuously.” (Lemoine 1807)

Dampier only stayed long enough to make repairs on his ship before returning to Britain. It was not until Captain James Cook arrived in 1770 that Europeans recognised that Australia had potential for European settlement. Cook had a very different impression of the Aboriginal Australians he had encountered while travelling up the east coast of Australia:

“From what I have said of the Natives of New-Holland they may appear to some to be the most wretched people upon earth, but in reality they are far happier than we Europeans; being wholly unacquainted not only with the superfluous but the necessary conveniences so much sought after in Europe, they are happy in not knowing the use of them.” (Cook and Beaglehole 1968)

However, despite Cook’s positive impressions, he did not recognise the Aboriginal Australian inhabitants as owners of the land, believing it was a ‘terra nullius’, territory belonging to nobody, and was therefore free to settle and colonise by whoever claimed it. Noting the potential for farming and agriculture, Australia was claimed in the name of King George III.
Spiralling crime rates, and overcrowding of prisons, was one of the main reasons Britain had been searching for suitable land in which to establish penal colonies. Industrialisation had resulted in large numbers of people moving away from traditional rural areas to big cities where unemployment rates and poverty were high. Britain had considered other geographic areas, such as the west coast of Africa, however this region was ruled out as early contact with the Indigenous population had shown they could be combative. In contrast, the Aboriginal Australians had barely been seen, and the ones that had been seen were considered to be passive and friendly (Bateson 2004).

In 1788, Captain Arthur Phillip and the First Fleet landed at Sydney Cove under orders to establish a permanent British colony in New South Wales. The First Fleet consisted of convicts, crew and guards. This was followed by a Second Fleet and a Third Fleet of convicts in 1789 and 1791 respectively, and it was not until 1793 that the first free settlers began arriving. Other ships arrived on a regular basis with additional convict labour and supplies, ensuring the colony flourished and the gradual exploration and settlement of the continent could begin (Bateson 2004).

1.1.4 Aboriginal culture up until 1788

At the time of British arrival, Aboriginal Australian’s had rich and complex cultural and social traditions that had evolved over the tens of thousands of years of relative isolation. According to their beliefs, the physical environment of each area, their ‘Country’, and all living and non-living things within it, had been created by spiritual ancestors who had once travelled across the landscape. Boundaries of an individual’s Country are corroborated by Dreaming (the time that the ancestor spirits lived) creation stories. As is true of many Indigenous cultures worldwide, the land was not owned, instead Aboriginal Australians belonged to their land (Cowan 1989; Turner 1991; Grieves 2009; Kerwin 2010).

Complex and intricate kinship arrangements existed within groups, and determined societal expectations and obligations of each member. Aboriginal Australians lived in semi-nomadic, small family groups, to which connection was based on heritage, cultural obligations and familial responsibilities. As a predominantly nomadic society,
significance was placed on the maintenance of familial relationships, group connections and deep connections to Country (Berndt & Berndt, 1992; Broome, 1994, 2005).

In addition to these social traditions, Aboriginal Australians also had well established, complex trade routes and arrangements. The continent was traversed by trade routes, some of which are thought to have existed for more than 20,000 years, crossing linguistic, cultural and social boundaries. These paths were not only used for travel and trade, but also for communication. The extent of these networks is evident from intricately engraved pearl shell objects from the Kimberley but found in Ooldea in South Australia, and baler shell ornaments from Cape York Peninsula found in Western Australia, thousands of kilometres from where they were crafted (Mountford 1976).

It is important to note that much of what we currently know about the first 150 years of European settlement in Australia, and its effects on the Aboriginal Australian population, come entirely from written accounts from a European perspective. The Aboriginal perspective has only recently begun to be documented through oral histories, and from discoveries in archaeology and linguistics. However much has been lost over time.

1.1.5 British attitudes on arrival

At the time of British arrival, scientific debate centred around questions related to the relationship between race and phenotypic traits, and whether they indicated a single (monogenism) or multiple (polygenism) origin for humankind (Morton 1849; Knox 1850; Nott and Gliddon 1854; Darwin 1871). Many questioned whether those of non-European descent might represent a separate species (Morton 1849; Nott and Gliddon 1854).

Much of this debate was directed by long held religious beliefs and ingrained attitudes in which Europeans judged those of other cultures based on the accepted standards and customs of their own culture (Fforde 2013). As many of these non-European civilisations had their own cultural and religious practices, they were automatically seen as ‘primitive’ and ‘less evolved’(Fforde 2002). This led to a new path of research examining those who carried ‘primitive’ traits, with the comparison of the skulls of
anatomically modern and archaic humans and those from different population groups worldwide taking on new significance (Knox 1850; Fforde 2002; Anderson and Perrin 2008).

It was with these viewpoints in mind that the British arrived to Australia. On arrival they were faced with an environment that was completely alien and many European settlers were initially fascinated by the Aboriginal Australian culture and way of life, and collected large numbers of their artefacts and sacred objects as examples of ‘primitive’ culture (Ashcroft, et al. 2013). However, it was this perceived ‘primitivism’ that led to prejudiced and negative views of Aboriginal Australians, being seen as the 'lowest' order of human, and the Europeans fearing the possibility of future violent conflicts (McNiven and Russell 2005; Anderson and Perrin 2008). These misconceptions, with Aboriginal Australian manners and customs being so different to anything the British had encountered before, led to the desire to either eliminate them or to assimilate them into British culture and thereby ‘civilising’ them.

1.1.6 Conflict, disease and population changes

It is a common misconception that the entirety of the Australian continent was settled by Europeans on 26th January 1788. This process did not occur immediately, with settlement commencing on the eastern coast, with other regions being colonised unevenly in both space and time. For example, settlement in the state of Victoria was delayed until 1834, the Northern Territory settlement being achieved in 1869, while other areas were not settled till much later in the 20th century (Mulvaney and Kamminga 1999).

Aboriginal reactions to the presence of Europeans took two forms, passive or violent. British settlers found that Aboriginal Australians did not fence the lands they claimed were theirs, or farm it in the ways the British did and therefore they felt justified in claiming possession. This led to the open denial of any Aboriginal land claims. Consequently, land was taken to supply colonial settlers. Denying Aboriginal Australians access to lands which they felt were theirs by birth right, and something they felt spiritually connected to, inevitably led to resistance and conflict (Butlin 1984; Broome 1988; Connor 2002; Reynolds 2006). While it is estimated in total that
approximately 1000 to 3000 Europeans were killed during the early decades after settlement, probably ten times that number of Aboriginal Australians were killed in the same period (Reynolds 1987; Broome 1988).

In addition to death due to conflict, one of the biggest contributors to Aboriginal Australian population decline was through introduced disease. Due to their relative isolation over many millennia, Aboriginal Australians had not been exposed to smallpox, tuberculosis, influenza or venereal diseases (Campbell 2002). Smallpox (*Variola major*) spread through many areas of European settlement, with residents of Sydney witnessing an outbreak that killed half the local population in a single year, 1789 (Butlin 1993; Broome 2005). It is estimated that at least 20% of the total Indigenous population of Australia died shortly after European settlement (Kirk and Thorne 1976), resulting in a dramatic reduction in the size of the total Aboriginal Australian population in the first decades of the new colony.

There has been considerable debate regarding the composition and size of the Aboriginal Australian population at the time of European settlement, however we cannot know with any surety. Many estimates were based on European observations after settlement, and were of a population already in pronounced decline. Estimates of the Aboriginal Australian population size at time of European settlement have varied enormously. These estimates ranged from 227,000 and 314,500 (Radcliffe-Brown 1930; Davidson 1938; Jones 1970; Smith 1980) Smith 1980), but this figure was increased later to 750,000 (White and Mulvaney 1987). Whatever the original population size was, it fell to just 61,000 individuals being recorded in the 1921 Census (Kirk 1983).

Disruption to traditional life, through displacement, conflict and/or introduced disease(s) led to many Aboriginal Australians being forced to migrate away from their Country and kin. Many of these people moved to major population centres in search of food supplies and medical treatment. This disruption led to many Aboriginal Australians becoming ‘fringe dwellers’, living on the margins of European society as outsiders, with lower standards of health, hygiene and nutrition (McGrath 1995).
Further difficulties for Aboriginal Australian women, was the imbalance of the ratio of male to female colonists, estimated to be six to one (Oxley 1996; Bateson 2004). This imbalance was a matter of considerable concern to British authorities, and it was inevitable that Aboriginal women were targeted by white male convicts from the first day of arrival (Atkinson 1997).

1.1.7 Missions and settlements
By the 1800s, the Government began encouraging private citizens and religious institutions to set up systemised European-style missions for Aboriginal Australians (Colonial Office 1844; Harris 1990; Mitchell 2011; New South Wales Government - Office of Environment and Heritage 2012). This was seen as an efficient way to control illness and malnutrition, as well as to protect them from colonial violence and sexual exploitation. It was also seen as a way in which to force Aboriginal Australians to become better assimilated to European ways, through the teaching of ‘Christian ideals’ (Ferry 1979; Ellinghaus 2003). However, missions were heavily controlled environments where Aboriginal Australian culture and heritage was suppressed, a place in which speaking non-English languages was strictly forbidden, and movement heavily restricted (Elkin 1951; Broome 2005; van Holst Pellekaan 2013a).

These missions were a vital part of a deliberate and systematic policy that attempted to separate Aboriginal Australians: those of mixed descent or ‘troublemakers’ from traditional or ‘full descent’ Aboriginals. A benefit of this was once the Aboriginal people had been moved on, more land would be free for colonial development and the residents of the mission could be used as readily available labour pools (Elkin 1951; Ellinghaus 2003).

However, many Aboriginal Australian’s did not respond to this lack of freedom and religious teaching in the way in which the Europeans had anticipated, and it became clear that their attempts to ‘civilise’ and ‘educate’ them was failing (Ferry 1979; Ellinghaus 2003). This was generally accepted as a sign that Aboriginal Australians were of low intelligence, that they could not be civilised, and were therefore doomed to extinction. It was with this attitude in mind that future policies, designed at softening the harshness of their decline, were developed (Elkin 1951).
In 1837 a British Parliamentary Select Committee investigated the treatment of Aboriginal Australians, and other Indigenous people from British colonies, found that there had been ample evidence of injustice and cruelty in the way in which Aboriginal Australians had been treated (British and Foreign Aborigines Protection Society 1837). They highlighted the futility of forcing Aboriginal Australians to ‘become European’ when it will just result in further mistreatment:

“It is obviously unreasonable to expect that men habituated and attached to a roving, unsettled life, should abandon their wandering habits, and engage in agricultural pursuits, when the experience of every day is reminding them, that the cultivation of the soil will, in their case, prove only a preparatory step to its seizure by others”. (British and Foreign Aborigines Protection Society 1837)

They proposed it necessary for immediate legislative intervention in the form of an Aboriginal Protection Board that would be responsible for communicating directly with Aboriginal Australian communities, arranging appropriate education and training for the young, acting as coroners in the circumstances of death, and to determine a simple and uncomplicated code of conduct for them to follow (British and Foreign Aborigines Protection Society 1837).

The Aboriginal Protection Board was officially formed in 1869, and it was their job to set up unmanaged and managed reserves in which to place Aboriginal Australians (Human Rights and Equal Opportunity Commission 1997). Unmanaged reserves were essentially just designated areas of land set aside for Aboriginal Australians to camp on, usually in areas already favoured by Aboriginals, with little to no management by government authorities. Contrasting this were managed reserves, which were established and operated by appointed government officials. Schooling, rations and housing were all provided however, who could and couldn’t live there was tightly controlled, with many people being forcibly moved onto and off the reserves whenever it was deemed necessary (New South Wales Government - Office of Environment and Heritage 2012).
The ‘protection policies’ put in place by the Aboriginal Protection Board, particularly on
the reserves, were all encompassing (Harris 1990). The policies forbade Aboriginal
Australian people from entering towns without permission and/or permits, regulated
where employment might be sought, and heavily restricted relationships between
individuals (Human Rights and Equal Opportunity Commission 1997; Ellinghaus
2003). The co-habitation of Aboriginal women with non-Aboriginal men was strictly
prohibited and offenders were arrested. In these cases, Aboriginal Australian women
were moved to reserves some distance away (Ellinghaus 2003).

1.1.8 Stolen Generations
In 1886 the states of Victoria and Western Australia passed Acts of Parliament that
became commonly known as the ‘Half-Caste Act’. This extension of the Aboriginal
Protection Act sought to remove Aboriginal people of mixed descent (referred to at that
time as ‘half-castes’) from reserves and force them to assimilate into European society.
While restricted to two states initially, it was adopted by all states by the end of the 19th
century (Boucher 2015).

The Act, once implemented, gave the Chief Protector legal guardianship over every
Aboriginal person and ‘half-caste’ child. It was still believed that ‘full blood’ Aboriginal
Australians were going to die out, and that people of mixed descent could be more easily
assimilated, due to their lighter skin colour (Read 1981). These children were seen as
‘neglected’ or ‘unprotected’ and were removed from their families so they could be given
opportunities for a better life, away from the contaminating influence of Aboriginal
people (Read 1981; Robson 1983; Broome 2005). These children became known as the
‘Stolen Generation’ (Read 1981).

Most removed children were placed in institutional facilities operated by religious or
charitable organisations, while a number were fostered out to white families (Read
1981). Many were taught to reject their Indigenous culture and were told stories about
their parents having died or abandoned them. Commonly, institutions failed to keep
records of the children’s lives before admittance, and many still do not know their date
of birth, where they were born, what their real names were or who their parents were.
Removals occurred any time ‘neglect’ was suspected, though what neglect entailed was not clearly defined, nor something that had to be proven (Read 1981).

*When I was about twelve or thirteen years old I was taken to Moola Bulla.*
*That’s where I lost my Aboriginal ways. The Police came one day from Halls Creek when they were going on patrol to Lansdowne and found me, a half-caste child. The manager ... took me down to Fitzroy Crossing to wait for the mail truck from Derby to take me to Moola Bulla. When [the manager’s wife] told my people, mum and dad, that they were taking me to Fitzroy Crossing for a trip, they told her ‘you make sure you bring her back’. They did not know that I would never see them again (Testimony from the Human Rights Equal Opportunity Commission 1997).*

The exact number of children removed in this manner is unknown, with estimates being widely disputed. The *Bringing Them Home* report states that between one in three and one in ten Indigenous children were forcibly removed from their families (Human Rights and Equal Opportunity Commission 1997). Mixed descent children continued to be forcibly removed until the early 1970s, with the Aborigines Act being passed in 1967, ending 108 years of being under a ‘Protection Board’ (Broome 2005).

The consequences of the first 179 years, up until 1967, was that very few Aboriginal Australian people managed to escape the direct and indirect effects of legislation that controlled and governed their lives (McCorquodale 1987; Human Rights and Equal Opportunity Commission 1997). This oppression led to the loss of cultural heritage, language, the erosion of rights and destruction of families. The effects of this oppression continue to play out even today.

**1.2 Introduction to the problem**

**1.2.1 European superiority**

In addition to a history of dispossession and oppression, Aboriginal Australians have also had to contend with the disturbance and removal of familial and ancestral human remains. As discussed in a previous section (Section 1.1.5), at the time of arrival of the British, scientific debate had been centred on the analyses of human difference,
particularly of those thought to exhibit ‘primitive’ traits. After Charles Darwin published *The Origin of the Species* in 1859, increased interest in the morphological and anatomical features exhibited by non-European populations prompted a rush in the acquisition of remains from around the world (Fforde 2013). Large institutions, such as universities and museums, aimed to obtain representative collections of all human ‘races’ worldwide.

The remains of Aboriginal Australians were much sought after from the earliest days of European settlement. Much of what drove this interest can be found in the observations of early European explorers and colonists, and scientific studies such as those of Huxley (1863) and Malthus (1798). The concept of what it was to be ‘civilised’, ‘intelligent’ or ‘human’ was shaped predominantly by definitions which supported the believed superiority of Europeans (McNiven and Russell 2005). The disinterest of Aboriginal Australians in embracing European ways after settlement, coupled with the fact that many believed them different from any other race in ‘features, complexion, habits and language’ (Wilkes 1845), suggested to some that they may be the ‘missing link’ between archaic humans and Europeans, or apes and man (Turnbull 2017).

Huxley’s 1863 research linking skull size and mental superiority, or inferiority, suggested the skulls of Aboriginal Australians were the ‘lowest and most degraded in rank of any which can claim humanity’ and were ‘near the degraded type of the Neanderthal skull’ (Huxley 1863). Malthus, likewise, suggested that Aboriginal Australians from Tasmania were second from ‘the bottom of the scale of human beings’ (Malthus 1798). Being perceived as the ‘lowest’ order of mankind and facing possible extinction, their remains were highly valued and much sought after within scientific circles and their collection encouraged. The scientific demand for Aboriginal Australian skeletal material raised little moral concern over the methods used in obtaining them.

**1.2.2 Acquisition of remains**

Museum catalogues and university inventories (Williamson 1857; Flower 1907) show that the majority of Aboriginal remains had been taken from a number of places such
as cemeteries, burial places and sites of colonial violence (Fforde 2002). An 1891 article in the Sydney Mail (J.F.H 1891), tells the story of the exhumation of the remains of Cumbo Gunerah, a famous Aboriginal Australian war-chief from Gunnedah in north-eastern New South Wales, by Dr Edward Haynes, government medical officer and surgeon, and mayor of Gunnedah. Haynes had spent considerable time in the area and had heard stories of Cumbo Gunerah’s bravery and death, however the location of his burial site had remained secret. A few days before an old Aboriginal Australian woman’s death, Dr Haynes encouraged her to confide the location to him and once known exhumed the remains for study, before sending them to the Australian Museum for display (J.F.H 1891).

This was far from a single occurrence, with the collection of skulls and skeletal material from gravesites common. Another well documented example was that of Adelaide coroner William Ramsay Smith who made numerous visits to the burial grounds in Coorong and Raukkan in South Australia to obtain the remains of hundreds of Aboriginal Australians which were sent to the University of Edinburgh (Fforde 2013). In addition to remains that were deliberately removed from their burial sites, others were uncovered through construction or erosion and were also sent to overseas institutions.

However, many remains were taken before funerary rites, with soft tissue in particular, being collected from hospitals and morgues. From these locations the brains, genitals, limbs, complete heads and whole bodies of Aboriginal Australian people were obtained and sent to British institutions. For example, the Royal College of Surgeons of England were sent at least two complete Aboriginal Australian bodies, and at least two heads preserved in ‘spirit’ were sent to Cambridge University (Fforde 2002). Hermann Klaatsch, a German physical anthropologist, was able to acquire brains from Broome Hospital in northern Western Australia, as well as the skeletal material of deceased Aboriginal Australian prisoners from the morgue at Wyndham jail (Stehlik 1986).

One of the most famous cases of the desecration and mutilation of the remains of an Aboriginal Australian was the 1869 death of the ‘last full-blooded male Tasmanian’
William Lanne. Before Lanne’s remains were sent for burial a fight over his remains commenced between the Royal Society of Tasmania and the Royal College of Surgeons in England (Cove 1994; Petrow 1997), with both parties wanting to study the remains of what they thought were a dying population, headed for extinction. Applications were made for permission to take the body for scientific research. Due to Lanne’s status the Tasmanian Government promised to exhume them and provide them to the Royal Society of Tasmania after a short internment, in order to avoid scandal. However, before Lanne was buried Dr William Lodewyk Crowther (who later become Premier of Tasmania) removed his skull and replaced it with one from a deceased European for the Royal College of Surgeons (Ryan 1996), while the Royal Society removed his hands and feet (Petrow 1997; MacDonald 2005a, b).

Rumours of Lanne’s mutilation spread and at his burial many mourners demanded to see the body. These demands were denied. The rumours persisted and calls were made for his body to be exhumed. The Tasmanian Government, keen to hide what had happened, ordered police to protect the grave, however Lanne’s remains were taken before they arrived. What happened to Lanne’s remains is still unknown, however it is thought his skull may have ended up at the University of Edinburgh (Cove 1994).

Following the public outcry over the treatment of William Lanne’s remains, living Aboriginal Australians went to great lengths to avoid the same happening to them, attempting to prevent the collection of their own remains. Truganini, the ‘Last Tasmanian’, was so distressed by the prospect of her mutilated remains becoming scientific specimens on display in a museum that seven years before her death in 1876 she asked the Reverend Atkinson to ensure her remains were buried in the deepest part of the d’Entrecasteaux Channel in south east Tasmania (H.D. Atkinson, cited by Fforde 2004). She also pleaded with the Tasmanian authorities for a respectful and undisturbed burial. Sadly, these requests were ignored and within two years of burial, her remains were exhumed after a successful application by the Royal Society of Tasmania. Her skeletal remains were then put on display in the Hobart Museum where she remained until 1947, before public sentiment led the museum to store her remains in the basement until 1976.
1.2.3 Benefits and attitudes

Payment for the collection of Aboriginal Australian remains was uncommon, with most institutions preferring acquisitions be donated instead. However, some institutions such as the Australian Museum keenly encouraged the trade of Aboriginal Australian remains, paying collectors for any skeletal material collected (Turnbull 1991). Even without a cash incentive, there were considerable benefits for those who became involved in the collection of Aboriginal Australian remains. This was usually in the form of involvement and patronage with and from renowned scientists, and contributing to high-level and important scientific research (Fforde 2013).

It might be argued that collectors may not have known what they were doing was morally and ethically wrong, even by the standards of the time. They believed their actions were justified as their considerable contribution to science and the understanding of human evolution far outweighed any moral concerns (Fforde 2002), besides the Aboriginal Australians were a ‘dying lower race’ anyway. However, Aboriginal Australian feelings regarding their dead and the sanctity of gravesites was well recognised by Europeans, with recorded accounts of Aboriginal Australians taking considerable care to avoid burial places for fear of disturbing resting spirits (Turnbull 2002), and reports of Aboriginal Australians taking precautions to avoid becoming scientific specimens themselves (i.e. Truganini). There are also accounts of the concern and dismay of Aboriginal Australians while witnessing the disturbance and removal of the dead from gravesites. In some cases Aboriginal Australians reacted aggressively (Stehlik 1986).

“Luckily, we had remained unnoticed by the blacks during our grave violating enterprise. However, they must have soon noticed what had happened because, after we had finally stowed away our spoils on the boat and had continued with our journey, we wanted to stop at an appropriate place to pick up water; it had already got dark, when our blacks turned our attention to little flashes of light that started to appear in the thickets of the shore. These were the fire-sticks in the hands of the natives who followed us” (Klaatsh 1907, translated by Stehlik 1986)
This aggressive opposition appears to have been so common that The Royal Geographic Society warned collectors that they may face violent opposition while collecting remains (Fforde 2002).

In 1913, after high profile inquiries into the actions of medical professionals such as the Adelaide coroner, William Ramsay Smith, who was suspended from duty after charges of misuse of human remains (Turnbull 1991), laws were passed making it illegal to export Aboriginal Australian remains without a permit. However, remains continued to be taken from Australia illegally until the late 1940s. This was relatively easy to do as evidenced by Swedish zoologist, Eric Mjoberg, who led the first Swedish expedition into the Kimberley in 1910. Mjoberg who was known to observe Aboriginal burial ceremonies, later raided the gravesites and smuggled the remains out of Australia as "kangaroo bones" (Thompson 2013).

1.2.4 The repatriation debate
For the past fifty years’ Indigenous groups worldwide have campaigned to know where their ancestors remains are being held and to be given the opportunity of reburying them (Fforde 2004). In Australia, the beginnings of this repatriation debate emerged in the 1960s, coinciding with the recognition of Aboriginal Australian rights when the Aborigines Act of 1967 was passed. However, community requests for repatriation of Aboriginal Australian remains occurred as early as the beginning of the twentieth century (Fforde 2004).

Internationally, museums and other institutions continue to hold collections of Aboriginal Australian remains in their catalogues. Over many decades, there has been a concerted effort by the Aboriginal Australian people, as well as the Australian Government and various museums, to reach agreement that would enable these ancestral remains to be repatriated (Truscott 2006; Pickering 2015). To date there has been extensive literature published on the repatriation of remains to communities; the ethical, social and political motivations behind such requests, and debate regarding the complexities of ‘ownership’ of these collected remains (Webb 1987; Mulvaney 1991; Meighan 1992; Fforde 2002; Hubert and Fforde 2002; Turnbull 2002; Fforde 2004; Truscott 2006; Turnbull 2007).
It is irrefutable that the treatment of Aboriginal Australians during the period of European colonisation has caused considerable hurt and trauma that has persisted to the present day. It is therefore understandable that Aboriginal Australian communities whose ancestral remains were collected and displayed in museums across the world are requesting they be repatriated to their rightful home. This issue is of particular importance to Aboriginal Australians because of their spiritual connection to their land (Cowan 1989; Turner 1991; Grieves 2009; Kerwin 2010).

Connection to the land is a defining characteristic of Aboriginal Australian society (Kerwin 2010). It is believed that the land, and the seas, rivers, flora, fauna and people contained within it, were created through the activities of Spirit Ancestors during the Dreaming (Edwards 1994; Grieves 2009; Kerwin 2010). The spirits of these Ancestors are passed on to their descendants throughout time, and influence every aspect of Aboriginal Australian culture, spirituality, social responsibilities and kinship (Broome 1994; Bird 2003). This intricate interconnectedness links Aboriginal Australian individuals not only with each other, but also to the Dreaming and what is known as their ‘conception site’. A conception site is the place on the Dreaming track, or songline, in which a particular ancestral event took place, such as their birth. Aboriginal Australians retain lifelong associations with this site and many believe that they belong to this site and the land it exists in (Berndt 1974).

The connections that bring together kin, community and land, also bring with it a community responsibility to care for their ancestors (Broome 1994). Aboriginal Australians believe that for ancestral spirits to rest they must return to their ancestral lands and their kin, and re-enter the cycle of Dreaming, to become land once again (Bird 2003; Truscott 2006). This is termed a ‘return to Country’. With remains being stored within museums and not being returned to Country, Aboriginal Australians feel that they are failing in their social responsibilities for their ancestors, and until they are reburied there will likely be severe consequences for both the dead and living, and the wellbeing of their kin and community (Hubert 1989).

However, spiritual beliefs are not the only reason Aboriginal Australians are campaigning for the return of ancestral remains. Museums continuing to hold human
remains in their collection against the wishes of Aboriginal Australian communities are often seen as a continuation of the behaviour Aboriginal Australians encountered after European colonisation of the Australian continent (Fforde 2013). Many see this issue as just a smaller part of a much larger problem, that of historic injustices in the name of colonialism, loss of property rights and widespread oppression, all of which led to the collection of Aboriginal Australian ancestral remains in the first place (Hubert and Fforde 2002). Many Aboriginal Australians see repatriation as a way of acknowledging and attempting to correct these past injustices and allowing the healing process to begin (Truscott 2006). It would also allow their ancestors to finally rest in peace.

1.2.5 Repatriation and science

The repatriation of human remains from museum collections is a highly debated and complex issue for those working in museums, scientists and researchers. For museums, the failure to retain human remains, Aboriginal Australian or otherwise, in their collections can result in their inability to continue research. It has been argued that museums are designed by nature to hold historic material in their collections and protect it on behalf of future generations, and therefore human remains play a key role in their existence. Hence repatriation is in direct conflict with this (Jenkins 2011).

One of the major concerns of scientists and researchers about repatriation is the overall loss of significant scientific information that may be recovered through continued research on human remains (Turnbull 2002, 2007). These human remains have the potential to make major contributions to new scientific knowledge about human evolution, adaptation, population genetics, health and disease.

A notable example of the debate generated when scientifically important sets of Aboriginal Australian remains are earmarked for repatriation is that of the Kow Swamp and Lake Mungo remains. These remains are some of the most significant Aboriginal Australian remains discovered to date due to their age, with Mungo Man dating up to 42,000 years (Bowler, et al. 2003), and the remains displaying unique gracile and robust morphologies (Bulbeck 1998). In the early 1990s, when these remains were repatriated, many scientists publicly opposed Indigenous claims over remains housed in museums, and an intense debate occurred over what was seen as a
loss of vital scientific knowledge if they were reburied (Webb 1987; Zimmerman 1989; Mulvaney 1991; Meighan 1992; Gough 1996; Jones and Harris 1998; Zimmerman 2002; Lahn 2006; Pardoe 2012a).

One of the major criticisms to claims regarding the loss of scientific knowledge and the importance of human remains is that in many instances remains have spent considerable lengths of time in museum collections and have had no significant research completed or published (Hubert and Fforde 2002; Turnbull 2007). Many Aboriginal Australians feel that researchers have had the remains of their ancestors long enough. Researchers often counter these arguments by suggesting that retention of the remains will allow further study when new technologies are developed, and when new research questions and techniques emerge (Turnbull 2002).

Some scientists are a little more extreme in their views about repatriation. Some have strongly argued that repatriation as a process is anti-science and places the spiritual beliefs of a small section of society above the public interest of humanity as a whole (Meighan 1992; Stringer 2003). Others feel the loss of human remains that have been the topic of published research violates one of the most fundamental scientific principles involving reproducibility and the ability to replicate earlier research, damaging the integrity of scientific research (Working Group on Human Remains in Museum Collections 2003).

It is important to note that many museums and institutions insist their collections of human remains were obtained through legally acceptable ways such as donations and purchases, conforming to standards and values which were current at the time of their procurement (Pickering and Gordon 2011). It is for this reason that many museums have declined to provide information regarding the contents of their collections to enquiring Aboriginal Australians. Some institutions, such as the Duckworth Laboratory at Cambridge University, have very strict guidelines in place about the types of repatriation requests that they will consider. The Duckworth Laboratory will not accept repatriation requests based on merely geographic origin or cultural association. Only proven next of kin relatives of named individuals or those who can establish biological
relatedness with unnamed individuals will have their requests considered (The Leverhulme Centre for Human Evolutionary Studies 2008).

1.2.6 How are repatriation requests processed?

Repatriation requests are processed differently from institution to institution, with each having their own processes in place. There are commonalities, however each application is assessed on a case-by-case basis. Once a request is received from an individual or a community, it is essential that curators clarify the nature and scope of the request (Department for Culture Media and Sport 2005; Truscott 2006; Pickering and Gordon 2011; Pardoe 2013). This includes confirming the identity of the claimants and establishing a basis for the claim. For many institutions, a cultural or geographic connection is not sufficient, with claimants required to demonstrate that they have a direct and close genealogical link with the ancestral remains that they wish to repatriate (Department for Culture Media and Sport 2005). But given the turbulent past, this is extremely difficult to provide for individuals who died more than 100 years ago (Turnbull 2016). These individuals may have large numbers of descendants from more than one community.

Assessing repatriation requests can take a considerable amount of time, labour and money, as the provenance of each set of ancestral remains are researched thoroughly (Pardoe 2013; Fforde, et al. 2015). It is often a difficult and lengthy process, involving a number of community consultations. This process involves painstaking analyses of documentation such as historical records, reports, letters and diaries of collectors for clues about the origin of the remains in question (Fforde, et al. 2015; Turnbull 2016). Typically, museums will only repatriate after this archival research has been completed and they are confident that the ancestral remains are being returned to the correct geographic location and community (Pardoe 2013).

Even when the provenance of remains is known, these remains can sometimes be moved into unprovenanced categories after archival research has been completed. One example of how this can occur was documented by Deanne Hanchant, an archival researcher working on the National Skeletal Provenancing Program in Australia. Hanchant (2004) outlined a situation in which the Aboriginal Australian claimants of a
provenanced skull asked for further research to be undertaken in order to locate associated post-cranial remains of the same individual. Additional research established that the cranium had been donated by a donor from Tehore in India and marked as being from an “Australian Native”. Biological anthropologists were asked to examine the cranium, which was determined to be of probable Indian origin and not Aboriginal Australian at all. This additional research prevented the wrong individual being repatriated and reburied in Country it did not belong to (Hanchant 2004).

Additional complications in the repatriation process can occur when museums or other institutions receive claims for remains from more than one Aboriginal Australian community (Pickering 2001). Repatriation claims can become heavily contested with courts of law becoming involved in the process. In some circumstances judicial injunctions have been served, preventing the museum from repatriating the remains until a solution has been agreed to by all parties involved (McGlade 1998; Davies and Galloway 2009). Even when the repatriation process is straightforward, disputes within the claimant community may occur about what should happen to the remains, where they should be kept, and the proposed reburial (Pickering 2001).

1.2.7 Difficulties

While some ancestral remains have been, or are in the process of being returned to Aboriginal Australian communities, repatriation of many collections continues to be a complex issue. Many remains, estimated to between 20 and 25% of some Australian museum catalogues (Hanchant 2004; Truscott 2006; Pardoe 2013) but is likely much higher internationally, were collected with no specific detail of any kind regarding their geographic origins, tribal affiliations or language groups absent. For many, the only information provided was that they were ‘Aboriginal Australian’ in origin. Other remains were collected with accompanying documentation, however over time these details have been lost, were recorded incorrectly, or the remains themselves have been separated or stored in such a way that a number of individuals were stored in communal boxes (Truscott 2006; Pardoe 2013).

This lack of information regarding their provenance, means that many remains are unable to be returned to Country, with museums and institutions not willing to
repatriate without first identifying the appropriate communities or custodians (Turnbull 2007). Some overseas institutions have also said they are reluctant to return ancestral remains to Australia as they will not be returned to Aboriginal Australian peoples but would simply be stored indefinitely in a museum facility (Working Group on Human Remains in Museum Collections 2003). The National Museum of Australia currently house over 200 such remains in its care. These ancestral remains were returned from overseas institutions and are not accessioned into the collection of the receiving museum, and are kept on behalf of Aboriginal Australian People (Commonwealth of Australia 2015).

Given these conditions, they are unlikely to ever be studied due to their lack of context and they cannot be repatriated either, remaining in limbo, destined to continue being stored in museum storerooms. This means that for a large number of ancestral remains repatriation is not a simple issue as many Aboriginal communities do not want the remains unless it is known exactly where the remains come from (Truscott 2006).

Given the spiritual beliefs of Aboriginal Australians, and the expected consequences of burying ancestral remains in the incorrect Country, it is essential that the provenance of remains be as precise as possible. A well-publicised example of a problematic repatriation is that of the Murray Black Collection. George Murray Black, an amateur collector of Aboriginal remains collected very large numbers of remains during the 1930s to the 1950s (Prince 2015). The collection, the largest assemblage of Aboriginal Australian remains at the time of its collection comprised of approximately 1700 individuals from nine burial sites along the Murray River of New South Wales and Victoria. These remains ranged from 100 years to 10,000 years in age (Robertson 2007). Black neglected to keep records of the provenance of the remains, instead focussing on collecting remains he thought would be beneficial to research (Robertson 2007).

Black collected as many bones as he had crates to hold them. In 1940 he wrote to the Anatomy Institute: "I regret to say that through lack of cases I was unable to pack most of the specimens or to arrange for shipment… [I] packed 13 cases of skeletons and long bones leaving unpacked enough skeletons and long bones for about 12 cases… I dumped all the incomplete skeletons except long bones into the creek… as you appear
to only require complete skeletons.” (Pickering 2008). These remains were returned to the Aboriginal Australian people in 1989. Some were reburied close to their original resting place, while others were buried in the land of another tribal group, well away from their Country, resulting in conflict between neighbouring Aboriginal communities (Pardoe 1991b).

1.2.8 How are remains currently repatriated?

To date, repatriation in Australia has focussed predominantly on returning remains the provenance of which is known (Fforde 2002; Pickering 2015), or through identification by non- or minimally destructive methods such as craniometrics or stable isotope analyses (Pate, et al. 2002; Pardoe 2004). Craniometric methods can be used to test for population affinity using multivariate statistical analysis of cranial and post-cranial dimensions (Pardoe 2004). This allows for comparison against databases of worldwide craniometric measurements. The Remains Identification Program, has been successfully used to facilitate the repatriation of Aboriginal Australian remains to state or regional level (Pardoe 2004). However, this method is dependent on an extensive database of reference craniometric measurements for the population being available. Given the complexity of how remains are stored in museums (i.e. a number of individuals stored in communal boxes), this method would only be useful for the repatriation of crania, not the associated unprovenanced skeletal material.

Stable isotope analysis may also be used, with isotopes found in rocks, water, plants and animals being incorporated into the tissue of people from the food they eat and the water they drink (Makarewicz and Sealy 2015). Measuring the ratio of different isotopes potentially allows us to identify where a person was born or where they grew to adulthood (Pate, et al. 2002; Makarewicz and Sealy 2015). One weakness of this method however is that it requires very detailed mapping of regional isotopic base levels, and for many regions of Australia this information has not yet been collected.

To date, the use of DNA has not been used for the repatriation of Aboriginal Australian remains. However, recent advances in ancient genomics, particularly with the identification and subsequent repatriation of the famous 8,500 year old Kennewick Man
(Rasmussen, Sikora, et al. 2015) suggest that DNA-based methods could be used to assist with the identification of unprovenanced Aboriginal Australian remains.

Though, given the traumatic history of Aboriginal Australians, especially in regards to the collection of ancestral remains, it is not surprising that many contemporary Aboriginal Australians are wary of scientific research. These feelings have been further exacerbated by research that suggested that contemporary Aboriginal Australians were not the First People of Australia, and the unethical treatment of contemporary research participants in larger genomics based research such as the Human Genome Diversity Project.

1.2.8.1 Vampire project

Some of the previous genetic research involving Indigenous communities across the world have been contentious. While such research can often involve the study of disease or genetic traits, the majority of the research relates to population genetics with the aim of determining the genetic history of groups involved, and how they relate to other populations around the world. One of the most contentious studies involving Indigenous populations, including Aboriginal Australians, was the Human Genome Diversity Project (HGDP) begun in the 1990s (Cavalli-Sforza, et al. 1991). The aim of the research was to collect DNA from Indigenous groups in order to understand the diversity of humans all over the world. It created considerable controversy by referring to the targeted Indigenous populations as “isolates of historical interest”, while outlining the need for samples before they became extinct or were assimilated (Roberts 1992; Dodson and Williamson 1999; Reardon 2005).

“Isolated human populations contain much more informative genetic records than more recent, urban ones. Such isolated human populations are being rapidly merged with their neighbours, however, destroying irrevocably the information needed to reconstruct our evolutionary history. Population growth, famine, war, and improvements in transportation and communication are encroaching on once stable populations. It would be tragically ironic if, during the same decade that biological tools for
understanding our species were created, major opportunities for applying them were squandered” (Cavalli-Sforza, et al. 1991).

This description it was argued indicated the researchers saw these Indigenous populations as little more than historical curiosities (Reardon 2005).

Much of the opposition, with the strongest coming from Aboriginal Australians, was that Indigenous peoples were being subjected to ‘bioprospecting’ or ‘biopiracy’ (Callaway 2011). Key concerns of the ‘Vampire Project’, as it became known by its critics due to its focus on blood samples, was that the genetic samples could be modified and patented, before becoming commercially valuable without informed consent (Australian Human Rights Commission 2002). If this occurred, the Indigenous people themselves would receive little or no recognition for their contribution, despite the prospect of their possible extinction or assimilation being highlighted in key HGDP discussions (Reardon 2005).

As a consequence of this, many feel that science continues to see Aboriginal Australians more as subjects than research partners. This has led to many Aboriginal Australian communities being reluctant, even opposed, to genetic research as a general rule, as it is potentially a continuation of the ‘Vampire Project’.

1.2.8.2 Adcock et al. controversy

Additionally, the findings of the 2001 study (Adcock, et al. 2001) by Australian National University’s Gregory Adcock and colleagues was highly controversial amongst Aboriginal Australians. The authors published what was argued to be mitochondrial DNA sequences from Mungo Man, the oldest Aboriginal Australian from the Willandra Lakes region of New South Wales, and nine other ancient Aboriginal Australians.

The most significant claim made by the authors was that the ancient mitochondrial DNA sequence of Mungo Man was highly divergent when compared with the mitochondrial sequences of contemporary humans, including those of Aboriginal Australians. The authors suggested that Mungo Man therefore belonged to an early human lineage which predated, and was unrelated to, contemporary Aboriginal Australians (Adcock, et al. 2001). The implications of these claims were immense. The
authors suggested that there had been multiple waves of migration to Australia, with an
older more divergent population of which Mungo Man was a member, being replaced
by a more recent population consisting of the ancestors of present-day Aboriginal
Australians.

The suggestion that contemporary Aboriginal Australians were not the true First
Peoples of Australia resulted in heated debate and upset amongst Aboriginal
Australians. These controversial findings were challenged by other researchers who
questioned the authenticity of the sequences reported, with many questioning the
methods the authors had used to analyse the recovered DNA sequences and concerns
about the validity of the conclusions (Colgan 2001; Cooper, et al. 2001; Groves 2001;
Trueman 2001; Cameron and Groves 2004).

1.2.9 Changing tides

Many Aboriginal Australian communities remain sensitive to genetic research and have
been outspoken in their opposition of such research (Dodson 2000; van Holst Pellekaan
Aboriginal Australian communities are starting to see research involving both
archaeology and DNA as a means of giving them back some of the knowledge of their
past that recent history has denied them. Aboriginal Australians are becoming
genuinely curious about their genetic history and learning about how their ancestors
lived. Aboriginal Traditional Owners we have worked with have suggested that ancient
remains are making their way to the surface, and being discovered, because their
ancestors wish their stories to be told (Tapij Wales, personal communication, 23
October 2013).

Sensitive handling of the 2011 study (Rasmussen, et al. 2011) of a 100 year old lock of
hair of an Aboriginal Man from Western Australia, which resulted in the publication of
the first complete genome of an Aboriginal Australian, has led to more Aboriginal
Australians wishing to become involved in research about themselves, and actively
collaborating in scientific research.
We published two papers in collaboration with Aboriginal Australian communities in 2016. The first addressed the controversy generated by the conclusions presented by Adcock et al. (2001) (Heupink, et al. 2016b) (Appendix 1.1 - 1.4). Professor David Lambert and Dr Leon Huynen were given consent from the Willandra Lakes World Heritage Area Aboriginal Elders Committee, comprising the Paakantji, Ngiyampaa and Mutthi Mutthi Elders to resample a number of the original study’s ancient Aboriginal Australian remains. We provided strong evidence that the DNA sequences originally reported were likely polymerase chain reaction (PCR) artifacts, and did not originate from Aboriginal Australians. The study was important to the Aboriginal Australian communities involved as it refuted the offensive suggestion that contemporary Aboriginal Australians were not the First People of Australia.

The second study (Malaspinas, et al. 2016) (Appendix 1.5 – 1.9) undertaken in partnership with nine Aboriginal Australian communities across the Australian continent, to assess the genomic history of contemporary Aboriginal Australia. One of the most important aspects of this research was the inclusion of each Aboriginal Australian Traditional Owner as authors.

These papers helped change the way many Aboriginal Australians perceived scientific research. Rather than being mere scientific ‘subjects’, they were active partners and collaborators, involved in all aspects of the research and recognised publicly for their important contributions.

1.3 This research

The idea for this PhD research came about through conversations with Aboriginal Australian Traditional Owners. Initially the aim was to focus on unlocking the genomic secrets of ancient Aboriginal Australians, however the Traditional Owners expressed their interest in learning more about how the ancient Aboriginal Australians were genetically related to individuals from the contemporary community. It was at this time that our Aboriginal Australian research partners asked whether DNA might assist in them learning more about themselves. This could potentially then solve a major
problem and assist with the repatriation of Aboriginal Australian unprovenanced remains.

1.3.1 Community partnership
This research was conducted in collaboration and partnership with a number of Aboriginal Australian communities and their Traditional Owners. We consulted extensively with the Traditional Owners from each community regarding the nature and extent of the research. These groups specifically include:

- The Yidiny and Gimuy Walubara People of Cairns, Queensland
- The Kurnu Paakantji People of the Riverine, New South Wales
- The Thaynakwith People of Cape York
- The Paakantji, Ngiyampaa, and Mutthi Mutthi People of the Willandra Lakes, New South Wales
- The Barapa Barapa Nation of Barham / Koondrook-Perricoota, New South Wales
- The Gkuthaarn and Kukatk People of Normanton, Queensland
- The Cape Melville, Flinders & Howick Islands Aboriginal Corporation Group, Queensland

Aboriginal Australian partnership in this research was essential, without their perspective much of what we aimed to do would not have been possible.

1.3.2 Thesis objectives
Utilising both ancient and contemporary DNA methods this thesis will determine whether DNA can be used to facilitate the repatriation of unprovenanced ancestral Aboriginal Australian remains currently held in museums. The only method in which to test this is to use DNA recovered from ancestral remains of known origin, and use them as proxies for unprovenanced remains.

Four specific aims will assist in meeting this overall objective:

1. Determine whether it is possible to recover DNA from ancient Aboriginal Australian remains (Chapter 2).
2. Construct an appropriate reference dataset of contemporary Aboriginal Australian genomes (both mitochondrial and nuclear) with which to compare DNA from ancient samples (Chapter 3).

3. Determine whether it is possible to use mitochondrial DNA, and to what accuracy, to assist in the repatriation of ancient Aboriginal Australians against the reference dataset (Chapter 4).

4. Determine whether it is possible to use nuclear genomic sequences, and to what accuracy, to assist in the repatriation of ancient Aboriginal Australians (Chapter 5).

Through these aims it will also be possible to make observations about the genetic diversity of both ancient and contemporary Aboriginal Australians.

1.3.3 Potential difficulties, limitations and concerns

There are a number of potential difficulties, limitations and concerns that may arise throughout the duration of this research.

Firstly, recovery of ancient DNA is extremely difficult, more so given the hot and humid climate of the Australian continent (Hofreiter, et al. 2015). Ancient DNA recovery from pre-European aged human remains has been limited to one study (Heupink, et al. 2015) due to DNA degradation. However, there have been a number of successful instances of ancient DNA recovery from fossilised remains of megafaunal marsupials, birds and reptiles (Murray, et al. 2013; Haouchar, et al. 2014; Llamas, et al. 2015; Grealy, et al. 2016; Haouchar, et al. 2016; Brüniche-Olsen, et al. 2018; Thomson, et al. 2018; White, et al. 2018).

It won’t always be possible to recover DNA from poorly preserved human remains and even if DNA is able to be recovered, it is possible that the analyses will not result in the answers that Aboriginal Australians are hoping to receive. There may be no conclusive results due to the contemporary dataset being too small, not covering a sufficient geographical range, or because DNA is not able to be recovered from the individual sample itself.
Another serious concern is that this research could potentially cause harm or distress to Aboriginal Australian participants, and the Aboriginal community as a whole. Repatriation and DNA-based research are sensitive and controversial topics. While we have had a number of Aboriginal Australian communities happily agree to partner with us in this research, some groups have remained either sceptical or unhappy about scientists working with ancestral remains, especially given that it is by nature a destructive process. Naturally, in these cases, the research has not proceeded.

The results of the analyses completed may be confronting to some, and may contradict Aboriginal Australian traditional knowledge and narratives. However, in all aspects of this research we have been honest about the possible difficulties, limitations and expected end results. We will continue to communicate directly with the communities involved through informal conversations, official community meetings and plain language reporting.

1.3.4 Thesis structure

This PhD thesis is presented as a series of chapters, an unpublished manuscript that will be submitted to Science as a review article and associated appendices of work completed during my PhD candidature.

Chapter two presents background information regarding the sixty sets of ancient Aboriginal Australian remains analysed. It focuses specifically on the complexities associated with the recovery of ancient DNA, the problem with contamination and the need to authenticate recovered ancient DNA sequences.

Chapter three addresses what was required to construct meaningful contemporary Aboriginal Australian DNA datasets that were used in conjunction with the recovered ancient mitogenomes and nuclear genomes presented in Chapter Two. It discusses the different types of DNA that could be used, and the advantages and disadvantages of each. In this chapter, the background of the new contemporary genomes are discussed and outlines the novel mitogenomic haplotypes recovered.
Chapter four specifically answers whether mitochondrial DNA can successfully assist with provenancing ancient individuals, facilitating their repatriation. This is completed using phylogenetics, with novel haplotypes presented.

Chapter Five is presented in the form of a manuscript, submitted for publication. This manuscript contains analyses presented in chapters two, three and four, but also includes new nuclear analyses to determine whether it is possible to use DNA to repatriate unprovenanced Aboriginal Australian remains.

Chapter Six discusses whether the results can be seen as a new model for repatriation in Australia, the complexity involved with decision-making and problematic returns of ancestral material. It makes recommendations for the direction of future research, and discusses the significance of the research completed.

The appendices presented in this thesis include two published papers with associated social impact material. The first paper, ‘Ancient mtDNA sequences from the First Australians revisited’ was published on 21 June, 2016 in PNAS. This paper was publicised worldwide and was followed by a companion piece written for The Conversation. The second paper, ‘A genomic history of Aboriginal Australia’ was published on 21 September, 2016 in Nature. This paper was featured on the cover of Nature and was widely publicised around the world. This too was followed by a companion piece written for The Conversation, and was voted one of the top ten scientific ‘Breakthroughs of the Year’ for 2016 by the editors and writers of the journal Science.
Statement of contribution to co-authored published papers included in this thesis.

The following chapters include material from two co-authored papers. The bibliographic details of each co-authored published paper, including all authors, are detailed below:

**Paper 1:**

**NOTE:** Malaspinaas to Bergström are equal first authors; Athanasiadis to Wright are equal second authors (in alphabetical order). This paper could not be used as a complete chapter of this thesis as Griffith University Higher Degree of Research rules state that only first author papers may replace chapters, and I was a second author.

My contribution to the published paper involved:

- Collaborating with local groups to collect Aboriginal Australian samples. This including collecting saliva samples, holding discussions regarding ethics approvals and research outcomes with Traditional Owners and participants, and writing plain English reports of the research findings.
- Performed critical background research.
- Performed preliminary lab work on saliva samples before sequencing.
• Analysis of genetic data. I was involved in the analyses of both mitochondrial and nuclear DNA. This included phylogenetic analyses and f- and d-statistics (included in this thesis).
• I provided critical input to discussions and helped write the manuscript and supplementary from October 2013 until publication in September 2016.

Paper 2:

My contribution to the published paper involved:
• Performed critical background research.
• Analysis of mitochondrial data.
• I provided critical input to discussions, and helped write the manuscript and supplementary.

Original copies of the aforementioned papers can be found in Appendix 1 and 4.

_________________________________
Joanne Louise Wright

_________________________________
Principal supervisor & corresponding author of the published papers  Professor David M. Lambert
Chapter 2: Ancient Aboriginal Australian DNA

2.1 Aim of the chapter
This chapter will explain the nature of ancient DNA (aDNA): its characteristics, what factors can affect the amount of recoverable DNA, and the challenges this often presents. The precautions needed for working with ancient DNA will then be outlined together with an explanation of how these data can be used in repatriation studies. Finally, details will be presented of the ancient Aboriginal Australian DNA research completed as part of this PhD program.

2.2 The beginnings of the aDNA revolution
Ancient DNA has been broadly defined as the DNA sequences recovered from fossil remains, excavated archaeological finds, museum specimens and other unusual sources of DNA which date back decades to hundreds or thousands of years (Pääbo, et al. 2004). The study of aDNA began in 1984 with the sequencing of 229 base pairs of the mitogenome of a quagga, an extinct relative of both the zebra and horse (Higuchi, et al. 1984). This was soon followed with the publication of a cloned sequence from a 2.4ky old Egyptian mummy (Pääbo 1985). Though this ancient Egyptian sequence was later attributed to modern contamination (Del Pozzo and Guardiola 1989; Knapp, et al. 2015), these studies led to questions about what else could be learned through the analysis of aDNA. Until the first complete ancient human genome of a 4ky old Palaeo-Eskimo from the Saqqaq culture of Greenland was published in 2010 (Rasmussen, et al. 2010), many researchers thought obtaining a complete genome from ancient material was impossible. Since then however, there have been many studies investigating for example ancient extinct animal species (Orlando, et al. 2013; Heupink, et al. 2014; Grealy, et al. 2017; Westbury, et al. 2017); the evolutionary history of diseases, pathogens and bacteria (Pósa, et al. 2015; Rasmussen, Allentoft, et al. 2015); the origins of domestication (Ramos-Madrigal, et al. 2016; MacHugh, et al. 2017); and the population histories of humans, Neanderthals and Denisovans ( Sawyer, et al. 2015; Meyer, et al. 2016; Prüfer, et al. 2017; Sikora, et al. 2017).
The aDNA recovered in many of these earlier studies came from amplifying genomic DNA by PCR. However, this required large amounts of fossil material to be destroyed in order to sequence even partial and certainly a complete mitochondrial genome (Der Sarkissian, et al. 2015). In additional, the study of such remains was a massive investment for any researcher in terms of both workload and financial cost (Der Sarkissian, et al. 2015; Hofreiter, et al. 2015). In the past decade, however, a revolution in the aDNA field has occurred due to the advent of high-throughput and next generation sequencing technologies, allowing researchers to target shorter and more fragmented DNA. As a result, many remains that were once considered unsuitable were then successfully being sequenced.

2.3 The nature of aDNA

2.3.1 DNA decay

DNA decay starts immediately after death, when chemical and biological processes begin breaking down soft tissue. The decay process is driven by bacteria, fungi, and other microorganisms that feed on the body’s cells, breaking down membranes and cell walls. Once these cell walls are ruptured, catabolic enzymes stored within are released degrading and fragmenting DNA strands into shorter and shorter fragments over time (Lindahl 1993).

Variability in DNA survival is not only affected by these rapidly occurring chemical and biological processes. In addition, the physical environment in which the remains are interred can also play a crucial role. Water, temperature, humidity, soil pH and UV light can all affect the degradation process considerably, either accelerating or slowing down the rate of decay (Lindahl 1993; Hofreiter, et al. 2001).

Typically, environments with high temperatures, humidity, rainfall and acidic soils encourage faster DNA degradation rates. This occurs both through chemical and enzymatic processes, but also because these types of environments provide ideal conditions for bacterial growth. By far the most favourable environment for DNA survival is that of permafrost, in which conditions are cold and dry, slowing down the degradation process considerably (Lindahl 1993; Hofreiter, et al. 2001). In rare
circumstances, if soft tissue is rapidly desiccated after death, or the remains become calcified or mineralised, it may escape such enzymatic and microbial degradation somewhat (Pääbo, et al. 2004; Green and Speller 2017).

Hard tissue such as bone or teeth degrade at much slower rates than soft tissues due to their mineral composition, which protects their cells from enzymatic and environmental processes. Bones typically consist of two-thirds hydroxyapatite, a form of calcium phosphate which binds DNA, helping to slow down the degradation process over time (Briggs, et al. 2007; Brundin, et al. 2013). However, once the collagen component of hard tissue breaks down, temperature and environmental conditions increase bone porosity allowing these processes to accelerate again (Hedges 2002). As a general rule, aDNA from teeth is less prone to degradation than bone because it has been encased in enamel, the hardest and most highly mineralised substance of the body, protecting the internal dentine and pulp from external factors (Damgaard, et al. 2015; Glocke and Meyer 2017; Hansen, et al. 2017).

Another hard tissue that appears to preserve DNA better than any other part of the human skeleton are petrous bones. The petrous bone is part of the skull’s temporal bone where the inner ear is located, consisting of bony labyrinths, housing the structures responsible for both hearing and balance in humans. This temporal bone is relatively free of contamination due to its dense and closed nature (Misner, et al. 2009; Pinhasi, et al. 2015; Hansen, et al. 2017).

The end result of all of these processes is that DNA recovered from ancient remains exists in much smaller quantities and in shorter fragment lengths than might be expected from younger specimens (Hofreiter, et al. 2001; Pääbo, et al. 2004; Molak and Ho 2011). Characteristically aDNA shows a common pattern: fragments are limited to only a few hundred base pairs or less (Pääbo, et al. 2004); bases are lost resulting in abasic sites; and they contain blocking or miscoding lesions (Lindahl 1993; Höss, et al. 1996; Hofreiter, et al. 2001; Pääbo, et al. 2004; Willerslev and Cooper 2005). This damage occurs through oxidative and hydrolytic processes, regardless of environmental conditions (Lindahl 1993). Each of these damage types impacts aDNA recovery, requiring specialised laboratory techniques and downstream data analyses.
2.3.2 Fragmentation and abasic sites

Fragmentation of DNA occurs due to hydrolytic depurination, in which water breaks the bond between base-sugars and adenine or guanine bases, resulting in abasic sites (containing neither a purine nor a pyrimidine) (Lindahl 1993; Dabney, et al. 2013). Abasic sites can prevent DNA extension during the replication step of a PCR by obstructing DNA polymerases. This can lead to an amplification bias towards less damaged DNA fragments (Mouttham, et al. 2015).

With the advent of high-throughput sequencing, a better understanding of aDNA fragmentation occurred through the construction of DNA sequencing libraries. During this process it is necessary for adaptors to be ligated to the ends of DNA fragments in order for them to be sequenced (Dabney, et al. 2013; Mouttham, et al. 2015). As this type of damage occurs primarily at the 5’ end of DNA fragments, DNA library construction methods that utilise a blunt-end repair step, typically using a T4 DNA polymerase, have been found to rectify this problem. Blunt-end repair removes unpaired nucleotides from 3’ ends of DNA fragments (Briggs, et al. 2007) resulting in a blunt-end for adaptors to adhere to.

Another serious problem is the retention of very short DNA fragments (<50 bp). These fragments may be lost in the initial extraction and sequencing library construction limiting the recovery of endogenous aDNA. Many of the standard protocols for the recovery of ancient DNA involve laboratory clean up steps which rely on consumables such as the Qiagen MinElute PCR Purification silica-membrane spin columns (Rohland, et al. 2010). These silica membranes are designed to retain fragment lengths >70bp and remove fragments <40bp (Qiagen 2008), resulting in the loss of highly fragmented aDNA.

2.3.3 Miscoding lesions

Miscoding lesions occur primarily at the 5’ and 3’ ends of DNA, with cytosine bases deaminated to uracils (Dabney, et al. 2013). Uracils are not normally found in DNA, usually pairing with adenine and replacing thymine during RNA transcription. They generally only occur in DNA as a result of deamination, the loss of an amino group from a molecule due to hydrolytic processes (Sawyer, et al. 2012).
Uracil residues are problematic as they can reduce, inhibit or cause the misreading of DNA templates by some DNA polymerases. These DNA polymerases treat uracils as thymines, and insert an adenine on the opposite DNA strand. This produces incorrect C→T and G→A transitions (Lindahl 1993; Höss, et al. 1996; Hofreiter, et al. 2001; Pääbo, et al. 2004; Molak and Ho 2011). Therefore, an original CG base-pair is converted to a TA base pair in amplified DNA, impacting the accuracy of the recovered aDNA.

There are two methods for working with aDNA containing uracils. The first is to treat aDNA extracts with an enzyme called uracil-DNA-glycosylase (UDG). UDG removes uracil from DNA leaving behind an abasic site, so the opposite strand remains unpaired (Briggs, et al. 2007). This method improves sequence quality and decreases the risk of mistaking cytosine deamination for true mutations, but the downsides are that the already low levels of aDNA are further reduced, and the signature that may be used to authenticate aDNA sequences cannot be distinguished from modern contamination (Hofreiter, et al. 2001). Building two sequencing libraries from a single aDNA extract, one treated with UDG and one without, is the best approach to not losing this distinct measure of authenticity.

The other approach, and the one employed in this research, is to use a DNA polymerase specially designed to be used with uracil residues. KAPA HiFi HotStart Uracil+ is one example of a DNA polymerase that detects uracil residue in the template strand and allows it to be amplified, reducing amplification biases.

### 2.3.4 Blocking lesions

Blocking lesions occur in DNA in the form of nucleotide modifications such as oxidated pyrimidines (guanines in particular) and cross-links between DNA strands, fragments or other molecules such as proteins. A common characteristic of these sorts of DNA damage is that they obstruct the movement of DNA polymerases along template strands, preventing both PCR amplification and sequencing of DNA libraries. It is as though the DNA is not present.
2.4 Exogenous contamination and endogenous levels

One of the most serious issues affecting aDNA research is contemporary contamination. The main problem with this contamination is its relative abundance. The level of endogenous DNA (DNA from the subject itself) remaining in even a well preserved sample is vastly less than that of exogenous DNA (DNA derived from contamination). Depending on the source of the contamination, once incorporated into an ancient sample, it can overwhelm the aDNA of interest and bias any analyses (Serre, et al. 2004).

In many instances aDNA extracts can consist almost entirely of contamination. For the majority of ancient remains, it might be expected that the endogenous DNA content would fall somewhere between <1-5% of the total DNA of a sample, with 95-99% representing contamination (Burbano, et al. 2010; Green, et al. 2010; Stoneking and Krause 2011; Carpenter, et al. 2013).

The exception are remains discovered in permafrost, with the extreme cold temperatures helping to preserve much higher endogenous DNA levels. Notable studies of permafrost DNA preservation have included plants (Willerslev, et al. 2003), bison (Shapiro, et al. 2004), a 700,000 year old horse (Orlando, et al. 2013), microbes (Bellemain, et al. 2013), penguins (Lambert, et al. 2002; Ritchie, et al. 2004) and viruses (Legendre, et al. 2014).

As high-throughput sequencing does not discriminate between endogenous and contaminating sequences, the cost of sequencing a sample with just 1% endogenous DNA can make the recovery of a complete genome prohibitive for many research groups (Carpenter, et al. 2013). Additionally, low levels of endogenous DNA place some limitations on the types of analyses that can be completed, with many requiring minimum levels of coverage (Parks and Lambert 2015; Kousathanas, et al. 2017).

2.4.1 Environmental and human contamination

There are two main types of contamination: the first is environmental, and the second is human. Environmental contamination can originate from fungi, bacteria, microorganisms and animals that at one time made contact with the ancient remains (Hofreiter, et al. 2001; Pääbo, et al. 2004; Willerslev and Cooper 2005). While it can be
frustrating for researchers to waste both effort and funds sequencing large levels of contaminating DNA, if this contamination is evolutionarily different from the DNA of target, it does not pose too difficult a challenge in downstream analyses. It will be filtered out once the DNA sequences are aligned to a reference genome.

However, the most serious source of contamination, specifically for ancient human or archaic hominin specimens, is contamination from contemporary humans (Willerslev and Cooper 2005; Fulton 2012). Contamination in these types of samples is difficult to contend with due to the close evolutionary relationship between the remains and the researchers handling them or performing the analyses. This is particularly pronounced when the researchers share the same ancestry as the remains they are working on. It can be impossible to distinguish authentic aDNA sequences from human contamination once it is incorporated (Stoneking and Krause 2011).

Human contamination can occur a number of ways, from the time of initial excavation by archaeologists through to work completed in the aDNA laboratory (Hofreiter, et al. 2001; Willerslev and Cooper 2005). Every time remains are handled, contemporary human DNA is deposited on specimens and can be rapidly absorbed deep inside the bone through pores on the surface and cannot be removed by surface decontamination methods. This risk increases substantially when the bone is highly degraded, because the large pores and holes across the surface make the bone susceptible to deeper contamination (Pilli, et al. 2013). Although for some samples it is possible that the contamination is limited to the surface. Careful removal of the outer surface or sampling from the centre of the bone can result in less contaminated aDNA (Pilli, et al. 2013; Green and Speller 2017; Hansen, et al. 2017).

2.4.2 Limiting contamination in the aDNA laboratory

There is little that aDNA researchers can do about contamination that occurred prior to a samples arrival to the laboratory, other than to inform those collecting the samples of the dangers (Allentoft 2013). However, prevention of further contamination is very important and needs to be methodical and comprehensive. Many guides outlining methods to limit contamination in an aDNA laboratory have been published since the advent of aDNA (Pääbo, et al. 2004; Fulton 2012; Knapp, et al. 2012; Llamas, et al. 2017).
These methods fall broadly into three categories: a dedicated clean aDNA facility in
which to work with ancient remains, strict decontamination protocols and the use of
blanks and controls.

All aDNA work should be carried out in a laboratory dedicated exclusively for the
recovery of DNA from ancient or degraded samples and isolated from facilities in which
PCRs are completed or contemporary DNA processed (Knapp, et al. 2012; Llamas, et al.
2017). Ideally these facilities should be fitted with HEPA-filtered ventilation that is
positively pressured, preventing airflow from outside the facility (Knapp, et al. 2012).
The Griffith University aDNA facility has taken the additional precaution of sequencing
the mitochondrial genomes of researchers who have access to the aDNA in order to
determine whether human contamination detected, if any, originated within the facility.

Entrance to the aDNA facility should be restricted to all except the personnel
undertaking aDNA work. These researchers should wear disposable cleanroom
protective suits, surgical face masks, hair nets, shoe covers and two pairs of medical
gloves (one of which is regularly discarded during the work but certainly when working

Comprehensive and methodical decontamination methods should be applied when
working within the aDNA facility, or when new equipment enters the laboratory. These
methods include mechanical cleaning using >3% sodium hypochlorite and treatment
with UV irradiation ($\lambda = 254 \text{ nm}$) for a minimum of 30 minutes, but ideally longer
hypochlorite and UV light treatment is absolutely essential to prevent cross-
contamination between samples or experiments. Laboratory consumables and reagents
can also be a source of contamination, becoming contaminated during production in
the manufacturing facility (Leonard, et al. 2007; Champlot, et al. 2010). In this study,
additional precaution was taken of purchasing the highest grade laboratory reagents
(molecular grade) in order to mitigate this risk.

All aDNA work should include a number of blank controls (ideally extraction, DNA
library construction and PCR) to monitor the presence of contamination in reagents
used in the laboratory. These controls should include the chemicals used in extractions or library construction, but no DNA, and be routinely amplified and screened to ensure the reagents are contamination free (Rohland, et al. 2010). Even with all of these methods utilised, it by no means guarantees contamination will not be present once DNA libraries are sequenced.

2.4.3 Authenticating recovered aDNA

Because contamination in ancient samples is so ubiquitous, authenticating all DNA recovered after sequencing is essential. This can be done using a number of methods, and it is prudent to utilise as many of these as possible. The first is to utilise the characteristic C → T and G → A damage pattern of cytosine deamination, and the presence of uracils to authenticate aDNA sequences (Höss, et al. 1996; Hofreiter, et al. 2001). If this pattern is not observed in recovered sequences, they likely represent contamination. A software package that can be utilised to identify and quantify post-mortem DNA damage is mapDamage. This software uses Bayesian statistics to provide estimates of the average length of DNA overhangs and cytosine deamination rates (Jónsson, et al. 2013).

The fragment length distribution of recovered sequences can be used to authenticate aDNA. As discussed previously, after death DNA is fragmented into shorter and shorter fragments (Lindahl 1993). As such, it would be expected that authentic aDNA would be short, typically shorter than 100bp in length (Sawyer, et al. 2012). If the DNA recovered is longer than this, it may be contamination. However, it is important to note that this method would not be sufficient in isolation for authenticating recovered DNA. It has been shown that the fragment length distribution of contaminating DNA can be comparable to target endogenous aDNA (Krause, et al. 2010).

Calculating endogenous DNA levels can also give some indication of whether the recovered DNA may be contaminated. Unless the sample was recovered from permafrost, it would be expected that endogenous DNA levels would typically be between <1-5% (Burbano, et al. 2010; Green, et al. 2010; Stoneking and Krause 2011; Carpenter, et al. 2013). If an endogenous level >5% is calculated, the recovered sequences should be further screened for authenticity.
After DNA sequencing, bioinformatic tools can be utilised to estimate the proportion of contaminating reads present in DNA libraries and to separate out contamination. Software package Schmutzi uses an iterative approach to estimate contemporary human DNA contamination in ancient mitochondrial DNA sequencing using both sequence deamination patterns and fragment length distributions (Renaud, et al. 2015). Schmutzi is able to reconstruct endogenous mitochondrial genomes even when contamination rates are greater than 50%. One limitation of this software however, is that samples that have been well preserved and exhibit low levels of cytosine deamination may result in incorrect high estimates of contamination (Renaud, et al. 2015).

2.5 Previous ancient Aboriginal Australian DNA studies

There have been limited genomic studies of contemporary Aboriginal Australians, and even fewer involving ancient DNA. The entirety of this area of research consists of four publications: two of which focus on pre-European settlement aged Aboriginal Australian remains (Adcock, et al. 2001; Heupink, et al. 2016b), with the final two focussing on samples dated to after European settlement (Rasmussen, et al. 2011; Tobler, et al. 2017).

As previously discussed (section 1.2.8.2), the first genomic study of ancient Aboriginal Australians recovered ten complete ancient mitogenomes and presented a controversial hypothesis about human settlement of the Australian continent based on phylogenetic analyses (Adcock, et al. 2001). Fifteen years after the initial study (Adcock, et al. 2001), we revisited, resampled and reanalysed new sequence data (Heupink, et al. 2016b). We determined the recovered ancient mitogenomes were likely PCR artifacts and was not authentic Aboriginal Australian DNA. More importantly, we successfully recovered the complete mitogenome of an ancient Aboriginal Australian individual labelled WLH4. This was the first example, to our knowledge, of DNA recovery from ancient remains in an Australian archaeological context (Durband, et al. 2012; Heupink, et al. 2016b), proving that it was possible to recover aDNA from ancient Aboriginal Australian samples, despite the harsh climate.
In 2011 the first complete Aboriginal Australian nuclear genome was published (Rasmussen, et al. 2011). This sequence was recovered from a 100-year-old lock of hair of an Aboriginal Man from south Western Australia collected by Alfred Cort Haddon, a British anthropologist and ethnologist. Finally, there was the publication of 111 mitogenomes recovered from Aboriginal Australian hair samples collected from communities in Cherbourg, Queensland, and Point Pearce and Koonibba, South Australia between 1928 and 1939 (Tobler, et al. 2017). All but six of these hair samples were collected during Harvard and Adelaide Universities Anthropological Expeditions lead by Norman Tindale and Joseph Birdsell.

As such, the research presented here of sixty ancient Aboriginal Australian individuals is the first of its kind. The aDNA recovered, both mitochondrial and nuclear, contributes significantly to what is known about the genomic history of Aboriginal Australians.

2.6 Ethical consent and community permissions

All ancient Aboriginal Australian remains included in this research were provided to us directly by Aboriginal Australian communities and Traditional Owners. Some of these remains had been previously repatriated from Australian museums, while others were obtained during salvage excavations initiated and conducted by Aboriginal Australian communities, Australian Government departments and archaeologists after being discovered eroding from their resting places. We consulted extensively with the Traditional Owners regarding the nature and extent of all proposed research.

Written consent from Aboriginal Australian communities was obtained before work commenced on any ancient Aboriginal Australian remains. Separate Human Research Ethics approval was sought for these ancient remains from the GUHREC, but as they represented non-identified ancient remains they were ruled exempt from human ethics research review and approval (see Appendix 7: Exemption from ethical review – ancient remains for further information).
2.7 Ancient Aboriginal Australians: background

Sixty ancient Aboriginal Australian individuals were included in this study from different areas of Australia (Figure 3). Remains where aDNA was recovered were compared to the previously published genome from a hair sample from Western Australia (Rasmussen et al. 2011). For many of these remains we were provided with just enough skeletal material for us to conduct one or two DNA extractions before the remains were returned/reburied by their Aboriginal Australian communities.

Figure 3 | Locations of ancient Aboriginal Australian remains used in this study
Green circles represent samples worked on at Griffith University. The pink circle represents the 100-year old hair genome (Rasmussen et al. 2011)

2.7.1 Cairns, Queensland

The ancient Aboriginal Australian remains from Cairns were purchased in the early twentieth century from anthropologists by the Queensland Museum, and were repatriated to the Menmuny Museum in Yarrabah and the Yidindji/Gungandji peoples of Cairns in the early 1990s. Chief custodian of the remains, Yidindji Elder Gudjugudju
Fourmile of the Gimuy Walubara people, in conjunction with other Yidindji and Gungandji Elders, is an active partner in the research and gave permission for the remains to be sampled and analysed.

The remains comprise six individuals of mixed sex, some with associated post-cranial remains. Because of the comparatively late settlement of the Cairns region, in the late 1890s, the remains are not likely to have admixed with either Europeans or East Asians.

**PA86 and PA109**

Individuals PA86 (Figure 4) and PA109 (Figure 5) were collected from the Mulgrave District of Cairns, North Queensland. The crania were sold to the Queensland Museum by Joseph Campbell, former archdeacon and cotton farmer, as part of a collection of the remains of over twenty people and more than 2,800 artefacts including shields, baskets and spears, for the sum of thirty pounds on 28 September 1916 (Mather 1986; Branagan 1998). Due to limited bone material these remains could not be directly dated, however based on the taphonomic condition of these crania these ancient Aboriginal Australian remains are likely to date to between 120 and 150 years of age.

![Figure 4 | Front and side views of the crania of ancient Aboriginal Australian PA86](Image)

Photos: Professor David Lambert.
The red ribbon attached to this cranium is thought to be for ease of carrying, since these remains were carried by Aboriginal Australians as they moved from place to place. Photos: Professor David Lambert.

PA409

The PA409 collection consists of the remains of three individuals from the Russell River area of Cairns, Queensland, Australia (Figure 6). The remains were found with burial goods of a broken reed basket and pieces of bark, and were purchased in 1904 from anthropologist and former Southern Protector of Aboriginals for Queensland, Archibald Meston, by the Queensland Museum, as part of their ethnographic collection (Turnbull 2015; Robins 2016).

The mandible (left) and humerus (right), from the Russell River area of Cairns, Queensland. Photos: Professor David Lambert.
Due to insufficient bone material and to prevent causing significant damage to the remains direct dating only one sample of the PA409 individuals were dated. Dating was completed by the Aarhus AMS Centre, Department of Physics and Astronomy at the University of Aarhus, in Denmark. The radiocarbon dates ($^{14}$C) were calibrated in Calib 7.0.2 (Stuiver et al. 1993) using the Southern Hemisphere calibration curve (Hogg et al. 2013) and showed an approximate age of between 214 and 270 years (Table 1).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>$^{14}$C age</th>
<th>Calibrated date (95.4% probability range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA409 (1)</td>
<td>175 ± 28 BP</td>
<td>1747 - 1803 AD</td>
</tr>
</tbody>
</table>

PA8

Individual PA8 was also collected from the Mulgrave District of Cairns, North Queensland by Joseph Campbell, former archdeacon and cotton farmer, and sold to the Queensland Museum on 21 October 1916. The crania of PA8 (Figure 7) was part of a collection of over twenty sets of human remains including at least seventeen Aboriginal Australians from the Cairns district, as well as Papuan and New Zealand remains (Mather 1986; Branagan 1998).

Figure 7  | Front and side views of the crania of ancient Aboriginal Australian PA8
           | Photos: Professor David Lambert.


2.7.2 Bourke, New South Wales

In 2012 the remains of an ancient Aboriginal Australian man (BK1) were found eroding from the bank of the Darling River, Toorale National Park, approximately 50km south west of Bourke, north-west New South Wales, in the country of the Kurnu Paakantji Aboriginal people. The Traditional Owners refer to the person as ‘Kaakutja’ (pronounced Kaa-koo-gee), a Paakantji word meaning ‘Older Brother’. The burial was exceptionally well preserved, with the positioning of Kaakutja’s remains indicating that he had received a respectful internment after what appeared to be a violent death (Figure 8). The way in which he died was the subject of an Australian television documentary (Catalyst: Toorale Man Murder Mystery, 2015) and subsequent publication (Westaway, et al. 2016).

Two samples of the skeletal remains were sent to Dr Rachel Wood, Radiocarbon Dating Laboratory at the Australian National University, for radiocarbon dating ($^{14}$C) (Fallon, et al. 2010); a metatarsal bone from the left foot and an incisor. The radiocarbon dates were calibrated against SHCal13 (Hogg, et al. 2013) or Bomb 13 SH 1_2 (Hua, et al. 2013) in OxCal v.4.2 (Ramsey 2009). These dates suggest that Kaakutja was killed ~700 years BP (Table 2).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>$^{14}$C age</th>
<th>Calibrated date (95.4% probability range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK1 Metatarsal (1.1)</td>
<td>745 ± 20 BP</td>
<td>1260-1280 AD</td>
</tr>
<tr>
<td>BK1 Metatarsal (1.2)</td>
<td>740 ± 20 BP</td>
<td></td>
</tr>
<tr>
<td>BK1 Metatarsal (2)</td>
<td>750 ± 22 BP</td>
<td></td>
</tr>
<tr>
<td>BK1 Incisor</td>
<td>765 ± 19 BP</td>
<td>1220 – 1280 AD</td>
</tr>
</tbody>
</table>

A single M3 (right upper) wisdom tooth was provided for aDNA analysis (Figure 8). The tooth was extremely well preserved and took quite a lot of manual effort in preparing it for extraction.
Figure 8 | The skeletal remains and tooth of ancient Aboriginal Australian Kaakutja
The skeletal remains, in situ, at time of excavation (left). The M3, right upper, wisdom tooth used for ancient DNA extraction (right). Photos: Dr Michael Westaway (left) & authors own (right).

2.7.3 Willandra Lakes, New South Wales
The Willandra Lakes series represents one of the most significant archaeological collections of ancient human remains in Australia. These ancient Aboriginal Australian remains were collected throughout the 1970s and 1980s, with the earliest discovery being made in 1968 (Bowler, et al. 1970). Consisting of over one hundred individuals (Webb 1989), the series represents one of the largest collections of late Pleistocene modern human remains in the world. It has previously been argued that two distinct genetic signatures are represented in this series, one representing an extinct lineage of
Homo sapiens (Adcock, et al. 2001), however this was later shown to be likely modern contaminants or PCR artifacts (Heupink, et al. 2016b).

Professor David Lambert and Dr Leon Huynen were given permission to work on the remains of twenty-two ancient Aboriginal Australians by the Willandra Lakes Council of Elders, the Elders of the Paakantji and Ngiyampaa People and the Mutthi Mutthi People (locations of these individuals, if known, are shown in Figure 9).

WLH4

Biological anthropologist and archaeologist Steve Webb reports the remains of ancient Aboriginal Australia WLH4 were found at the ‘Walls of China 3’ site at Lake Mungo in 1974, and belonged to a female (Webb 1989).
The preservation of ancient individual WLH4 was different to the other individuals in that the physical remains are not heavily mineralised (Figure 10). Although there are no radiocarbon dates for this burial, the individual would appear to be late Holocene in age. The distinct pattern of dental wear supports the view that this individual subsisted on a hunter-gatherer diet (Durband, et al. 2012).

![Figure 10](image)

**Figure 10** | The left tibia of ancient Aboriginal Australian WLH4
The photo shows bone taken for ancient DNA recovery. Photo: Dr Leon Huynen.

**Other Willandra Lakes remains**
We also sampled 22 individuals in addition to WLH4 from the Willandra Lakes series (Table 3).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age (ky BP)</th>
<th>Material used for DNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WLH3 (LM3 or ‘Mungo Man’)</td>
<td>38-42 (^{O})</td>
<td>Humerus</td>
</tr>
<tr>
<td>WLH11</td>
<td>Undetermined</td>
<td>Powdered bone</td>
</tr>
<tr>
<td>WLH15</td>
<td>0.1 (^{C})</td>
<td>Femur</td>
</tr>
<tr>
<td>WLH18</td>
<td>18.5-22.1 (^{U})</td>
<td>Unspecified bone</td>
</tr>
<tr>
<td>WLH19</td>
<td>10-17 (^{S})</td>
<td>Unspecified bone</td>
</tr>
<tr>
<td>WLH22</td>
<td>Undetermined</td>
<td>Unspecified bone</td>
</tr>
<tr>
<td>Sample ID</td>
<td>Age (ky BP)</td>
<td>Material used for DNA extraction</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>WLH24</td>
<td>Undetermined</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH28</td>
<td>8-13 C</td>
<td>Vertebrae</td>
</tr>
<tr>
<td>WLH45</td>
<td>10-19 S</td>
<td>Femur</td>
</tr>
<tr>
<td>WLH50</td>
<td>15-32 S</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH52</td>
<td>0.1 U</td>
<td>Unspecified bone</td>
</tr>
<tr>
<td>WLH55</td>
<td>3-5 C, 6-37.6 U</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH67</td>
<td>10-20 S</td>
<td>Femur</td>
</tr>
<tr>
<td>WLH68</td>
<td>10-20 S</td>
<td>Cranial fragment</td>
</tr>
<tr>
<td>WLH69</td>
<td>10-20 S</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH72</td>
<td>Undetermined</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH73</td>
<td>Undetermined</td>
<td>Unspecified bone</td>
</tr>
<tr>
<td>WLH101</td>
<td>Undetermined</td>
<td>Cranial fragment</td>
</tr>
<tr>
<td>WLH124</td>
<td>Undetermined</td>
<td>Tibia</td>
</tr>
<tr>
<td>WLH130</td>
<td>Undetermined</td>
<td>Unspecified bone</td>
</tr>
</tbody>
</table>

C = age determined by radiocarbon dating (Gillespie 2002)
S = age estimate from stratigraphic information (Webb 1989)
U = age determined by U/Th alpha spectrometry (Gillespie 2002)
O = age determined by optically stimulated luminescence (Bowler, et al. 2003)

2.7.4 Barham (Koondrook-Perricoota), New South Wales

These two sets of ancient Aboriginal Australian remains were discovered during construction of the Koondrook–Perricoota Forest Flood Enhancement Project, on opposite sides of Barber’s Creek Overflow. After discovery, research into these remains was requested by representatives of the Barapa Barapa Nation. While the two burials were found in similar locations, and are of similar antiquity, they differ in many other respects, such as ages at death and sex (Pardoe 2012b). The remains of these two individuals were provided to us by Dr Colin Pardoe, of Colin Pardoe Bio-Anthropology and Archaeology on behalf of the Barapa Barapa People.
KP1

The ancient remains of KP1 (Figure 11) were found tightly bound in a shroud. The way in which the remains had been arranged indicated that they had been wrapped with knees to chin and hands below their jaw, then lowered into a small, deep hole. Indicators of their age at the time of death were the caps of the limb bones that had only recently fused, indicating that growth had only recently finished, and the bones of the skull that had not yet started to fuse together. The ancient male individual KP1 had visible tooth wear characteristic of Barapa Barapa People who chew Wongal (a tall reed-like plant with strap-like leaves), to prepare fibrous string for nets, bags, and all the other items. Teeth of people from this region typically show the effects of this practice (Pardoe 2012b).

![Figure 11](image)

**Figure 11** | The remain and skull of ancient Aboriginal Australian KP1

The skeletal remains of KP1, in situ, at time of excavation (left). The crania of KP1 (right). Photos: Dr Colin Pardoe

Radiocarbon dating was completed by the Aarhus AMS Centre, Department of Physics and Astronomy at the University of Aarhus, in Denmark. The radiocarbon dates were calibrated in Calib 7.0.2 (Stuiver and Pearson 1993) using the Southern Hemisphere calibration curve (Hogg, et al. 2013) (Table 4) and showed an approximate date of 1600 years BP.
Table 4 | Calibrated radiocarbon ages for KP1.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>14C</th>
<th>SD</th>
<th>Calibrated Age BP (2σ)</th>
<th>Relative Area</th>
<th>Average Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP1</td>
<td>1692</td>
<td>28</td>
<td>1433-1441</td>
<td>1%</td>
<td>1441.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1456-1462</td>
<td>1%</td>
<td>1462.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1468-1611</td>
<td>97%</td>
<td>1611.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1670-1670</td>
<td>0%</td>
<td>1670.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1676-1692</td>
<td>2%</td>
<td>1692.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weighted average:</td>
<td></td>
<td>1540.6</td>
</tr>
</tbody>
</table>

KP2

This ancient Aboriginal Australian female was found buried on her side in a flexed position (Figure 12). This is one of the more common burial positions, particularly for women. She had been buried with a thick layer of bark lining the bottom of the grave. While this bark lining was well preserved, the bones were not. In recent decades, termites and plant roots had invaded the grave and many of the bones had been damaged as a consequence, leaving just fragments. Teeth are however generally more resistant to decay over time, and it was possible to get an idea of the amount of tooth wear through life. This woman’s teeth showed signs of wear typical of a diet where food was cooked in the ashes, and where Wongal was chewed to produce fibre for string (Pardoe 2012b).

Figure 12 | The remains of ancient Aboriginal Australian KP2
A diagram showing a typical flexed burial position (left). Tooth wear of KP2 with the first molar at the top that is most heavily worn (right). Diagram and photo: Dr Colin Pardoe
Radiocarbon dating was completed by the Aarhus AMS Centre, Department of Physics and Astronomy at the University of Aarhus, in Denmark. The radiocarbon dates were calibrated in Calib 7.0.2 (Stuiver and Pearson 1993) using the Southern Hemisphere calibration curve (Hogg, et al. 2013) (Table 5) and showed an approximate age of 1400 years BP.

Table 5 | Calibrated radiocarbon ages for KP2

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>14C</th>
<th>SD</th>
<th>Calibrated Age ranges BP (2σ)</th>
<th>Relative Area</th>
<th>Average Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP2</td>
<td>1616</td>
<td>28</td>
<td>1380-1390</td>
<td>2%</td>
<td>1390.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1402-1535</td>
<td>98%</td>
<td>1535.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weighted average: 1466.6</td>
</tr>
</tbody>
</table>

2.7.5 Weipa and Mapoon, Queensland

Duyfken Point, Weipa

The Duyfken Point ancient Aboriginal Australian remains found immediately on the coastline and situated within an eroding foredune, were first investigated by the Queensland Police in October 2012. The police determined that the remains were Aboriginal and left them in place. The Queensland Museum were subsequently contacted on behalf of the relevant Traditional Owners by Mr Dale Furley, Ranger Coordinator Napranum, Weipa. The Traditional Owners were concerned about the imminent destruction of the burial site due to the approaching wet season and requested assistance with the recovery and relocation of the remains (Westaway and Nichols 2013).
The Queensland Museum dispatched Dr Michael Westaway in January 2013 to assist the community in the salvage excavation of the remains. He was accompanied by Dr Stephen Nichols of the Cultural Heritage Unit, Department of Aboriginal and Torres Strait Islander and Multicultural Affairs. The burial site was located close to Duyfken Point, approximately 30km west of Weipa, Cape York Peninsula, Queensland, with a shell midden located approximately 1m above the unit in which the burial was located (Westaway and Nichols 2013).

![Figure 13](image1.jpg)  
Figure 13 | The remains of ancient Aboriginal Australian Duyfken 1  
The remains, in situ, exposed in late December 2012. Photos: Dr Michael Westaway.

The remains consisted of the upper part of the body, which included the skull, most of the right arm, some of the left arm, and parts of the collar bone. There was no question that the remains were that of an Aboriginal person, obvious from the context of the burial (buried beneath a shell midden) but there are also characteristics in the skull that indicate that the person is Aboriginal. There was a lot of wear on the top (occlusal) surface of the teeth (Figure 14), which tells us this person lived on a diet that was not heavily processed and probably incorporated a bit of grit and sand. In some of the larger teeth the molars have all the crowns worn flat (Westaway and Nichols 2013).
The teeth, thought to belong to a mature adult female, were dated using a combination of radiocarbon dating and OSL dating to over 3,000 years ago. Initial radiocarbon dating on a phalange failed due to insufficient collagen. Subsequent radiocarbon dating was completed on two shells collected from the shell midden associated with the burial, one collected from below the remains and one above.

The radiocarbon dates ($^{14}$C) were calibrated in Calib 7.0.2 (Stuiver and Pearson 1993) using the Southern Hemisphere calibration curve (Hogg, et al. 2013) (Table 6).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>14C</th>
<th>SD</th>
<th>Calibrated age ranges BP (2σ)</th>
<th>Average age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duyfken (seashell above)</td>
<td>2370</td>
<td>25</td>
<td>1681-2206</td>
<td>2206</td>
</tr>
<tr>
<td>Duyfken (seashell below)</td>
<td>3580</td>
<td>30</td>
<td>3156-3669</td>
<td>3669</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Average:</td>
<td>2937.5</td>
</tr>
</tbody>
</table>

Mapoon and Weipa

The hair samples included in this research were originally collected by Reverend Nikolaus Johann Hey, a German Moravian missionary who arrived in Mapoon on the west Cape York Peninsula on 28th November 1891. Hey established and oversaw the Batavia River Mission (later known as the Mapoon Mission) on behalf of the Presbyterian Church of Australia with Queensland Government financial assistance,
until his retirement in October 1919 (Wharton 1996). The Weipa Mission was established in 1898. The Mapoon and Weipa Missions had been established to protect the local Aboriginal Australian population from mistreatment and raids from the pearling and bêche-de-mer (sea cucumbers) industries (Queensland 1899; Kidd 1997).

The 18 hair samples were collected during Reverend Hey’s time at the Mapoon Mission, placing them between 98 and 126 years in age. These samples were sold or donated to the Queensland Museum (see Table 7 for details and Figure 15), who repatriated them back to the community. The hair samples were provided to us for analyses by Thaynakwith Traditional Elder Tapij Wales.

Table 7 | Background information for each of the hair samples

<table>
<thead>
<tr>
<th>Queensland Museum code</th>
<th>Sample ID</th>
<th>Details</th>
<th>Location</th>
<th>Tribe</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QE223-24</td>
<td>MH1</td>
<td>Female aged 25</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.25</td>
</tr>
<tr>
<td>QE223-31</td>
<td>MH2</td>
<td>Male aged 12</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.28</td>
</tr>
<tr>
<td>QE223-5</td>
<td>MH3</td>
<td>Female aged 26</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.40</td>
</tr>
<tr>
<td>Q223-29</td>
<td>MH4</td>
<td>Female aged 52</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.12</td>
</tr>
<tr>
<td>QE223-17</td>
<td>MH5</td>
<td>Female aged 42</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.45</td>
</tr>
<tr>
<td>QE223-16</td>
<td>MH6</td>
<td>Female aged 34</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.15</td>
</tr>
<tr>
<td>QE223-33</td>
<td>MH7</td>
<td>Male aged 7</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.21</td>
</tr>
<tr>
<td>QE223-30</td>
<td>MH8</td>
<td>Male aged 15</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.31</td>
</tr>
<tr>
<td>QE223-7</td>
<td>MH9</td>
<td>Female aged 42</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.32</td>
</tr>
<tr>
<td>QE223-20</td>
<td>MH10</td>
<td>Male aged 49</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.25</td>
</tr>
<tr>
<td>QE223-8</td>
<td>MH11</td>
<td>Female aged 39</td>
<td>Pennefather River, North Queensland</td>
<td>Nggerikudi</td>
<td>0.50</td>
</tr>
<tr>
<td>QE223-10</td>
<td>WPAH1</td>
<td>Female aged 39</td>
<td>Pine River, North Queensland</td>
<td>Winda Winda</td>
<td>0.40</td>
</tr>
<tr>
<td>QE223-14</td>
<td>WPAH2</td>
<td>Female aged 33</td>
<td>Pine River, North Queensland</td>
<td>Winda Winda</td>
<td>0.32</td>
</tr>
<tr>
<td>QE223-13</td>
<td>WPAH3</td>
<td>Female aged 34</td>
<td>Pine River, North Queensland</td>
<td>Winda Winda</td>
<td>0.21</td>
</tr>
<tr>
<td>QE223-3</td>
<td>WPAH4</td>
<td>Female aged 28</td>
<td>Pine River, North Queensland</td>
<td>Winda Winda</td>
<td>0.33</td>
</tr>
<tr>
<td>QE223-21</td>
<td>WPAH5</td>
<td>Female aged 42</td>
<td>Pine River, North Queensland</td>
<td>Winda Winda</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Queensland Museum code | Sample ID | Details | Location | Tribe | Weight (g)
--- | --- | --- | --- | --- | ---
QE223-6 | WPAH6 | Female aged 38 | Embley River, North Queensland | Winda Winda | 0.32
QE223-18 | WPAH7 | Female aged 41 | Embley River, North Queensland | Winda Winda | 0.29

Figure 15 | Queensland Museum information cards for all Mapoon and Weipa ancient Aboriginal Australian individuals

(A) left to right – MH1-MH3, (B) left to right – MH4-MH6, (C) left to right – MH7-MH9, (D) left to right – MH10, MH11 & WPAH1, (E) left to right – WPAH2-WPAH4, (F) left to right – WPAH5-WPAH7.
2.7.6 Normanton, Queensland

In 2015, Dr Michael Westaway was contacted by the Coronial Support Unit after a number of skeletal remains were found eroding on the southern outskirts of Normanton, a small town at the western base of the Gulf of Carpentaria, Queensland. The material was in an advanced state of degradation due to erosion and weathering (Westaway and Adams 2015).

Police confirmed the presence of eight individual crania, most likely Aboriginal Australian in origin. The burial site indicated that they were not an original burial and likely represented reburied repatriated human remains from a museum collection. This was later confirmed by Dr Stephen Nichols, a Queensland archaeologist with the Department of Aboriginal and Torres Strait Islander Partnerships (DATSIP), who contacted the Australian Museum for clarification (Westaway and Adams 2015).

The community were concerned that further deterioration of the remains would occur and requested assistance with the recovery and relocation of the remains. In September 2015, Griffith University PhD students David McGahan and Shaun Adams, accompanied by Dr Michael Westaway and Australian Museum senior conservator Dr Colin Macgregor, flew to Normanton for the excavation and reburial (Westaway and Adams 2015).

Figure 16 | Ancient Aboriginal Australian remains from Normanton
Photo: Shaun Adams.
Human remains exposed on the surface showed eight crania left to right in two rows. Tibia and fibula can be seen in the top left hand corner (Figure 16).

European settlers established the township of Normanton in 1868. In the years that followed, the area opened up to mining and farming, bringing with it a large influx of European and Chinese migrants, leading to conflict with the Aboriginal Australian inhabitants, and epidemics of infectious disease (Roth 1903). With the onset of disease Dr Walter Roth was appointed government medical officer in Normanton in 1896. Roth, a physician and anthropologist, arrived with an interest in Aboriginal culture and in biological anthropology.

The human remains from Normanton were individuals who had died of infectious disease at the Normanton Hospital where Roth was surgeon. They were subsequently transported to Sydney where they became part of the Australian Museum’s anthropological collection. It is thought that the remains were collected by Roth in the late 1890’s and sold to the Australian Museum in the early 20th century. Based on Australian Museum manifests, the remains date to between 1895 and 1902 consisting mostly of patients who had died of infectious diseases in Normanton Hospital (Westaway and Adams 2015).

The remains were provided to us for ancient DNA analyses by Dr Michael Westaway on behalf of the Carpentaria Land Council Aboriginal Corporation (CLCAC), the Normanton Rangers and the Gkuthaarn and Kukatk Traditional Owners.

2.7.7 Birdsville, Queensland

The ancient Birdsville Aboriginal Australian remains, aBDV1 and aBDV2, consist of two crania originally collected by anthropologist Norman Tindale in 1936 on Alton Downs Station in north-eastern South Australia. The remains were repatriated from the South Australian Museum to the Queensland Museum in 2010 (manifest listing (A57847 and A57848)), as the remains originated in Wangkangurru Country, QLD. The collection location is approximately 92 km southwest of Birdsville.

The general condition of the bones was poor and fragmentary, suggesting they had been exposed for a long period (Figure 17). An associated report written by Dr Steve Webb,
an archaeologist based at Bond University and consultant for the Australian Museum, suggests the remains are likely to be those of two males and possibly dating to several thousand years old (Webb n.d.).

Figure 17 | Ancient Aboriginal Australian remains from Birdsville

The navicular bone (left). Mandible containing molars (right).

The remains were provided to us for ancient DNA analyses by Colleen Wall, a Senior Dauwa Kabi Woman and representative of the Wangkangurru People.

2.7.8 Stanley Island, Queensland

In October 2015, Dr Michael Westaway was contacted by Sargent Moseley of the Queensland Police Forensics Unit to provide information about a set of skeletal remains found eroding from sand on Stanley Island, an island at the tip of Cape Melville in the Great Barrier Reef Marine Park in Queensland (Figure 18).

After consultation with Traditional Owner, Clarence Flinders, the Department of Aboriginal and Torres Strait Islander Partnerships and the Queensland Parks and Wildlife Service, it was decided a site visit be made before the arrival of the monsoonal rains and king tides. Fieldwork was conducted by members of the Stanley Island community, Rex and John Flinders, Danny Gordon, and Griffith University PhD students, David McGahan and Shaun Adams. Based on morphometric analyses conducted by Dr Michael Westaway it was determined that the remains belonged to a lightly built Aboriginal/ Torres Strait Islander female between 16 and 22 years of age.
Figure 18 | Exposed remains of an ancient Aboriginal Australian from Stanley Island
Photo: Shaun Adams.

A single tooth was sent to Dr Rachel Wood at the Radiocarbon Dating Laboratory at the Australian National University for radiocarbon dating (Fallon, et al. 2010). The radiocarbon dates were calibrated against SHCal13 (Hogg, et al. 2013) in OxCal v.4.2 (Ramsey 2009) (Table 8) and showed an approximate age of between 350-400 years BP.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>$^{14}$C age</th>
<th>Calibrated date (95.4% probability range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STI1 tooth</td>
<td>375 ± 25 BP</td>
<td>1544 – 1698 AD</td>
</tr>
</tbody>
</table>

A single tooth was provided for ancient DNA analyses by Dr Michael Westaway on behalf of Traditional Owner Clarence Flinders and the Stanley Island community. We labelled this sample STI.

2.7.9 Flinders Island, Queensland

Human skeletal remains were found on Flinders Island, at the tip of Cape Melville in the Great Barrier Reef Marine Park in Queensland in 2015. The remains of the Flinders Island individual were inspected by police anthropologist Donna MacGregor, accompanied by the Queensland Police Forensic Unit and Traditional Owner, Danny Gordon. Based on cranial morphological traits, it was determined the remains belonged
to a male Aboriginal/Torres Strait Islander of 40-50 years of age. Due to the type of burial and lack of trauma it was thought the remains may represent a pre- or near-contact time frame and was referred to the Department of Aboriginal and Torres Strait Islander Partnerships.

In consultation with the Traditional Owners it was decided that a rescue excavation take place before the wet season to prevent further erosion. A team of archaeologists, under the supervision of Dr Michael Westaway, and assisted by the Flinders Island Aboriginal community, undertook the excavation.

![Figure 19 | Burial excavation of an ancient Aboriginal Australian from Flinders Island](image)

Photo: Shaun Adams.

A sample of the skeletal remains (FLI2) were sent to Dr Rachel Wood at the Radiocarbon Dating Laboratory at the Australian National University for radiocarbon dating (Fallon, et al. 2010). The radiocarbon dates were calibrated against SHCal13 (Hogg, et al. 2013) in OxCal v.4.2 (Ramsey 2009) (Table 9) and showed an approximate age of between 350-400 years BP.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>^14C age</th>
<th>Calibrated date (95.4% probability range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLI2 tooth</td>
<td>435 ± 22 BP</td>
<td>1513 – 1682 AD</td>
</tr>
</tbody>
</table>
A single incisor tooth was provided to us for ancient DNA analyses by Dr Michael Westaway on behalf of Traditional Owner Danny Gordon and the Flinders Island community.

2.8 Ancient genomic methods
The overall aim for all sixty ancient Aboriginal Australian individuals was to recover complete nuclear genomes wherever possible. After preliminary shotgun sequencing it was possible to determine which remains would allow the recovery of mitochondrial or nuclear genomes, and which samples contained very little or no recoverable DNA.

2.8.1 DNA extraction
All pre-PCR procedures were carried out in the dedicated clean lab facilities at the Ancient DNA Facility of the Australian Research Centre of Human Evolution, Griffith University. This facility consists of three distinct interconnected rooms for sample storage, DNA extraction and library construction. The facility is sealed, geographically isolated from any modern molecular laboratory, has one directional airflow under positive pressure and is HEPA filtered. The skeletal remains and hair samples were processed within a UV sterilised UPLA-filtered vertical laminar flow cabinet (used for this purpose only).

Each sample was initially treated with 10% sodium hypochloride to remove any surface contaminants. Any remaining sodium hypochloride was removed from skeletal material with a wash of UltraPure DNase/RNase-Free Distilled Water (Invitrogen).

Skeletal material was processed using a Dremel rotary tool with a high speed diamond cutter head, or manually with a sterilised scalpel blade, with the outer surface discarded. DNA was extracted from ~50mg of bone or teeth powder following the protocol outlined in Heupink, et al. (2016b). Extraction blanks were included throughout all procedures.

Hair samples were processed in 2 to 4 mL of digestion buffer as described in Rasmussen, et al. (2011). This was incubated in a rotating incubator oven for 24 hours at 45 °C. After complete digestion, the samples were centrifuged at 12,000 rpm for 3 min. The
supernatant was combined with 10x volume of a modified binding buffer (500 mL of PB buffer (Qiagen), 1:250 pH indicator I (Qiagen), 15 mL 3M pH5.2 of NaOAC (Thermo Fisher) and 1.25 mL 5M NaCl (Invitrogen)). Extractions were cleaned using the MinElute Reaction Cleanup Kit (Qiagen) following the manufacturer’s protocol and eluted off the column using 100 µL EB buffer (Qiagen) after incubation for 10 min at 37 °C. Extraction blanks were included throughout all procedures.

2.8.2 DNA library construction methods

Double-stranded Illumina DNA Libraries were built according to the methods described by Meyer and Kircher (2010), Rasmussen, et al. (2011) and Heupink, et al. (2016b). Using the NEBNext DNA Library Prep Master Mix Set for 454 (New England Biolabs ref: E6070) 21.25μl of DNA extract was subjected to a quarter volume NEBNext End Repair Enzyme Mix. After a MinElute (Qiagen) purification with 10x binding buffer PB (Qiagen) the resulting solution was subjected to NEBNext Quick Ligation in a half volume reaction with 25μM blunt end Illumina specific adapters P5 and P7 (Integrated DNA Technologies). After an additional MinElute purification, the DNA was subjected to a half volume NEBNext Adapter Fill-In reaction.

The DNA libraries were amplified to levels required for sequencing using one of three Taq polymerase protocols: AccuPrime Pfx (Life Technologies), KAPA HiFi Hotstart Uracil (Kapa Biosystems) and Platinum Taq DNA Polymerase High Fidelity (Life Technologies). Where necessary a secondary PCR was used to increase quantities. For all three protocols the primary 100μl library PCR was done according to manufacturer’s instructions using 10 μM Illumina primer InPE1.0 and 10 μM Illumina indexing primers. The primary PCR was subsequently cleaned using the MinElute PCR Purification Kit (Qiagen) according to manufacturer’s instructions.

The secondary PCR used 5μl of the purified primary PCR and 10 μM primers IS5 and IS6 (Integrated DNA Technologies). The volume of the secondary PCR reaction was 33μl for the AccuPrime Pfx SuperMix (Life Technologies) protocol, cycling was as follows for both PCR stages: 95°C 10 min, cycling [95°C 15s, 60°C 30s, 68°C 30s], 10°C indefinitely. The volume of the secondary PCR reaction was 50μl for the KAPA HiFi HotStart Uracil+ ReadyMix (Kapa Biosystems) protocol, cycling was as follows for both
PCR stages: 98°C 2 min, cycling [98°C 30s, 60°C 15s, 72°C 15s], 72°C 1 min, 10°C indefinitely. The volume of the secondary PCR reaction was 50μl for the Platinum Taq DNA Polymerase High Fidelity (Life Technologies) protocol, cycling was as follows for both PCR stages: 94°C 1 min, cycling [94°C 15s, 60°C 30s, 68°C 30s], 10°C indefinitely.

Amplified libraries were quantified using the Bioanalyzer 2100 (Agilent Technologies) using a High-Sensitivity DNA chip, following the manufacturer’s instructions. This step was necessary to ensure that DNA length distributions did not show any significant artifacts from amplification (e.g. artificially long molecules due to serial binding or primer dimers), where these problems occurred the PCR amplification the cycle number or primer concentration was adjusted. All PCR and extraction blanks were screened for contaminant library constructs on the Bioanalyzer. Blank controls were processed with every library step and each PCR.

2.8.3 Whole-genome in-solution target capture
Between 100 and 500 ng of library amplified DNA was generated for each sample as described above using multiple secondary amplifications and additional libraries, some of which was kept aside for direct sequencing. DNA libraries were subjected to MYbaits whole human genome capture (MYcroarray) African male option. The target capture was performed according to manufacturer’s instructions with the following modifications: hybridisation was performed for 36 hrs at between 55°C and 57°C and the bead binding buffers, and initial 30 min incubation and further cleaning were also performed at the same hybridisation temperature. Post-capture libraries were amplified on beads using HiFi HotStart Uracil+ ReadyMix (Kapa Biosystems) according to the MYbaits manual (version 3) for between 14 and 17 cycles.

2.8.4 Ancient sequencing
Ancient samples were 100 base pair single end sequenced on the HiSeq 2500 Sequencing System (Illumina) by The Danish National High-Throughput DNA Sequencing Centre in Copenhagen or on the MiSeq Sequencing System (Illumina) using 150 v3 kits at the Griffith University DNA Sequencing Facility. Sequences processed by The Danish National High-Throughput DNA Sequencing Centre were base-called using CASAVA
1.8.2 (Illumina), demultiplexed and FASTQ file generated by the sequencing facility. MiSeq sequences were base-called, demultiplexed and FASTQ file generated using BaseSpace, Illumina’s genomic analysis platform (Illumina).

2.8.5 Ancient read processing

After sequencing, the FASTQ files were processed by removing the adapter sequences and checked for read quality. Adapter sequences were trimmed using fastx_clipper, part of FASTX-Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/), with reads shorter than 30 bases and low quality bases removed using parameters -Q 33 –l 30.

Reads were aligned to the human reference build GRCh37/hg19 for the nuclear genome or to the revised Cambridge Reference Sequence (rCRS) (accession number NC_012920.1 Andrews, et al. (1999)) for mitochondrial data using BWA 0.6.2-r126 software (Li and Durbin 2010) with the following options: seed disabled (Schubert 2012) and terminal low quality trimming (using parameter -q15). Duplicate reads were removed by the MarkDuplicates tool from the Picard 1.68 tools package (http://broadinstitute.github.io/picard/). The mapped reads were sorted, indexed and merged using SAMtools 0.1.18 (Li, et al. 2009; Li 2011a). The consensus mitogenome was generated using the SAMtools bcftools view –cg -command and converted to FASTA via SAMtools/bcftools/vcfutils (Li, et al. 2009).

2.8.6 Sex determination

Sex determination of all ancient Aboriginal Australian individuals was inferred using the method outlined in Skoglund, et al. (2013), utilising the provided Python script and discarding alignments with a mapping quality score of <30. The software computes the number of alignments to the Y chromosome as a fraction of the total alignments to both sex chromosomes, even when the sequences show contamination and characteristic aDNA damage patterns (Skoglund, et al. 2013).
2.9 Ancient genomic results and discussion

2.9.1 aDNA recovery: authentication and summary statistics

All recovered DNA sequences were authenticated using a number of methods. Firstly, DNA damage patterns were estimated for each sample using MapDamage software (Jónsson, et al. 2013). All samples exhibited damage patterns characteristic of ancient DNA, with elevated levels of cytosine to thymine misincorporations in the 5’ end of fragments, and guanine to adenine misincorporations in the 3’ end (Dabney, et al. 2013) (see Table 10 – columns CtoT and GtoA).

Contamination estimates were calculated using Schmutzi software (Renaud, et al. 2015) using the contDeam command which estimates contamination levels using deamination patterns and endogenous consensus sequences were generated using default settings. Both the Schmutzi generated consensus sequences and the original ancient sequences were then manually checked using the SAMtools tview command (Li, et al. 2009). Missing sites were replaced with “N”.

Endogenous levels and the mean read length also indicated the DNA recovered was likely authentic (see Table 10 – Endogenous Content and Mean Read Length).

Recovered sequences were also consistent with Aboriginal Australian mitochondrial haplotypes and did not match those carried by any of the ancient DNA laboratory members.

2.9.2 aDNA recovery: successes and failures

Where the quantity of skeletal material allowed, multiple attempts were made to recover aDNA from all sixty ancient Aboriginal Australian individuals. Some remains only allowed a single attempt at aDNA recovery, with additional skeletal material no longer available due to reburial. We gratefully accepted remains in whatever quantity and quality the collaborating Aboriginal Australian communities felt comfortable providing us, and we endeavoured to be as economical with the skeletal material as we could.

It was possible to recover aDNA from thirty of the original sixty ancient Aboriginal Australians (50%) (Table 10). Of these thirty, we successfully recovered nuclear genomes of nine individuals (30%) dated between 98 and 1600 years in age, ranging
between 0.3 and 6.9x coverage. Additionally, we were able to recover thirty complete mitogenomes dating between 98 and 1600 years in age, ranging between 2.3 and 331.9x coverage.

All samples for which DNA was recovered were analysed to determine whether a correlation existed between age, geographic location/climate or material type, and the levels of mean read length and endogenous DNA observed (Table 10). The mean read lengths ranged from 49.2 to 97.4 bp, and endogenous DNA ranged from 0.6 to 87.6%, with no apparent pattern in terms of age, geographic location/climate or material type. This means that it would be impossible to predict whether a sample would be a good candidate for DNA recovery based on these factors alone.
Table 10 | Summary statistics for successful ancient Aboriginal Australian samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Location</th>
<th>Sample Type</th>
<th>Age (years)</th>
<th>Polymerase Used</th>
<th>Mapped Reads&lt;sup&gt;1&lt;/sup&gt;</th>
<th>MtdNA Coverage</th>
<th>Nuclear Coverage</th>
<th>Endogenous Content (%)</th>
<th>Mean Read Length (bp)</th>
<th>CtoT&lt;sup&gt;2&lt;/sup&gt;</th>
<th>GtoA&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Sex&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BK1</td>
<td>Bourke, NSW</td>
<td>Bone</td>
<td>700</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>3,153,084</td>
<td>2.6</td>
<td>0.0</td>
<td>1.3</td>
<td>55.9</td>
<td>0.45</td>
<td>0.27</td>
<td>XY</td>
</tr>
<tr>
<td>FL12</td>
<td>Flinders Island, QLD</td>
<td>Tooth</td>
<td>335-504</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>88,090,951</td>
<td>66.9</td>
<td>0.3</td>
<td>14.6</td>
<td>72.6</td>
<td>0.12</td>
<td>0.09</td>
<td>XY</td>
</tr>
<tr>
<td>KP1</td>
<td>Barham, NSW</td>
<td>Tooth</td>
<td>~1600</td>
<td>AccuPrime Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>3,294,622,679</td>
<td>331.9</td>
<td>6.9</td>
<td>18.3</td>
<td>80.5</td>
<td>0.16</td>
<td>0.09</td>
<td>XY</td>
</tr>
<tr>
<td>KP2</td>
<td>Barham, NSW</td>
<td>Tooth</td>
<td>~1400</td>
<td>AccuPrime Kapa HiFi Uracil+</td>
<td>421,358,942</td>
<td>91.8</td>
<td>0.4</td>
<td>1.4</td>
<td>67.5</td>
<td>0.41</td>
<td>0.32</td>
<td>XX</td>
</tr>
<tr>
<td>MH1</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>5,449,356</td>
<td>32.8</td>
<td>0.0</td>
<td>37.3</td>
<td>66.9</td>
<td>0.07</td>
<td>0.09</td>
<td>XX</td>
</tr>
<tr>
<td>MH2</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>3,475,551</td>
<td>63.1</td>
<td>0.0</td>
<td>64.5</td>
<td>54.7</td>
<td>0.10</td>
<td>0.10</td>
<td>XY</td>
</tr>
<tr>
<td>MH3</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>10,364,697</td>
<td>17.3</td>
<td>0.0</td>
<td>7.5</td>
<td>71.7</td>
<td>0.09</td>
<td>0.10</td>
<td>XX</td>
</tr>
<tr>
<td>MH4</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>5,185,873</td>
<td>19.0</td>
<td>0.0</td>
<td>3.4</td>
<td>61.4</td>
<td>0.06</td>
<td>0.06</td>
<td>XX</td>
</tr>
<tr>
<td>MH5</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>9,801,771</td>
<td>7.0</td>
<td>0.0</td>
<td>4.4</td>
<td>69.3</td>
<td>0.04</td>
<td>0.03</td>
<td>XX</td>
</tr>
<tr>
<td>MH6</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>112,173,428</td>
<td>136.4</td>
<td>0.8</td>
<td>47.1</td>
<td>51.7</td>
<td>0.06</td>
<td>0.05</td>
<td>XX</td>
</tr>
<tr>
<td>MH7</td>
<td>Mapoon, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>48,167,897</td>
<td>108.4</td>
<td>0.3</td>
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<td>0.06</td>
<td>XY</td>
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<tr>
<td>Sample ID</td>
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<td>Sample Type</td>
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<td>Polymerase Used</td>
<td>Mapped Reads</td>
<td>MtDNA Coverage</td>
<td>Nuclear Coverage</td>
<td>Endogenous Content (%)</td>
<td>Mean Read Length (bp)</td>
<td>CtoT²</td>
<td>GtoA³</td>
<td>Sex⁴</td>
</tr>
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<td>Bone</td>
<td>-</td>
<td>Kapa HiFi Uracil+Platinum Taq HiFi</td>
<td>83,303,303</td>
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<td>0.0</td>
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<td>71.6</td>
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<td>0.10</td>
<td>XY</td>
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<td>Bone</td>
<td>-</td>
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<td>67.2</td>
<td>0.08</td>
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<td>XY</td>
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<td>PA409.1</td>
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<td>Kapa HiFi Uracil+Platinum Taq HiFi</td>
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<td>0.04</td>
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</tr>
<tr>
<td>PA409.3</td>
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<td>Bone</td>
<td>-</td>
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<td>0.05</td>
<td>0.04</td>
<td>-</td>
</tr>
<tr>
<td>STI</td>
<td>Stanley Island, QLD</td>
<td>Tooth</td>
<td>350-400</td>
<td>Kapa HiFi Uracil+Platinum Taq HiFi</td>
<td>78,784,083</td>
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<tr>
<td>Sample ID</td>
<td>Location</td>
<td>Sample Type</td>
<td>Age (years)</td>
<td>Polymerase Used</td>
<td>Mapped Reads¹</td>
<td>MtDNA Coverage</td>
<td>Nuclear Coverage</td>
<td>Endogenous Content (%)</td>
<td>Mean Read Length (bp)</td>
<td>CtoT²</td>
<td>GtoA³</td>
<td>Sex⁴</td>
</tr>
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<td>-------</td>
</tr>
<tr>
<td>WPAH1</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>5,000,482</td>
<td>12.3</td>
<td>0.0</td>
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<td>66.8</td>
<td>0.09</td>
<td>0.08</td>
<td>XX</td>
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<tr>
<td>WPAH2</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>12,699,002</td>
<td>46.7</td>
<td>0.0</td>
<td>12.9</td>
<td>76.8</td>
<td>0.09</td>
<td>0.08</td>
<td>XX</td>
</tr>
<tr>
<td>WPAH3</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>4,199,344</td>
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<td>0.14</td>
<td>0.12</td>
<td>XX</td>
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<tr>
<td>WPAH4</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>99,130,123</td>
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<td>62.2</td>
<td>49.2</td>
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<td>0.09</td>
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</tr>
<tr>
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<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>2,522,663</td>
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<td>0.08</td>
<td>0.07</td>
<td>XX</td>
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<tr>
<td>WPAH6</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>6,652,016</td>
<td>30.8</td>
<td>0.0</td>
<td>12.7</td>
<td>67.0</td>
<td>0.09</td>
<td>0.09</td>
<td>XX</td>
</tr>
<tr>
<td>WPAH7</td>
<td>Weipa, QLD</td>
<td>Hair</td>
<td>98-126</td>
<td>Kapa HiFi Uracil+ Platinum Taq HiFi</td>
<td>6,449,981</td>
<td>12.4</td>
<td>0.0</td>
<td>12.8</td>
<td>72.1</td>
<td>0.11</td>
<td>0.07</td>
<td>XX</td>
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</tbody>
</table>

**Table key:**
1) Reads retained after adapter removal, fragment length filtering and quality filtering
2) Ratio of C-to-T substitutions at the most 5’ position of reads
3) Ratio of G-to-A substitutions at the most 3’ position of reads
4) Using method published by Skoglund et al. (2013). A dash indicates that sex was unable to be assigned
Of the thirty ancient Aboriginal Australian individuals for which aDNA recovery was unsuccessful (50%) (Table 11), the reasons behind these failures can be attributed to three main categories. The first is simply poor DNA preservation. A number of remains that we were provided by Aboriginal Australian communities had been discovered either eroding out and sitting on the surface, or among sand dunes. As previously discussed, UV light, heat, humidity and water all affect the DNA degradation process (Lindahl 1993; Hofreiter, et al. 2001) and Australia has an extremely hostile climate in this regard.

Indeed, many of the samples we were given, especially those from Normanton, Duyfken Point and Birdsville in Queensland, and those from Willandra Lakes, New South Wales, showed signs of advanced levels of degradation visually, with an almost complete breakdown of the bone leaving a chalk like mass behind. These geographic regions are known for their tropical (Normanton and Duyfken Point), desert (Birdsville) and semi-arid (Willandra Lakes) climate types with mean maximum temperatures in these areas ranging between 32.3 and 40.3°C during the summer months (Commonwealth of Australia - Bureau of Meterology 2017).

There were a number of instances in which the DNA recovered was contamination, displaying known European specific mitochondrial haplogroups H*, U* and K* (Torroni, et al. 2000). These haplogroups are not indicative of Aboriginal Australian DNA, which includes macrohaplogroup ‘M’ (Q* and M*), macrohaplogroup ‘N’ (O* and S*) and the ‘R’ subgroup of ‘N’ (P* and R*) (van Holst Pellekaan 2013b). As such, work on these samples was discontinued.

For a number of remains originating from Normanton, Queensland (NORA2-3, 5-8), it became apparent during the DNA extraction step that the remains had likely been chemically treated shortly after death or when accessioned into the museum collection. When the extraction buffer was added to the raw bone powder the solution unexpectedly turned a vibrant purple colour. It is possible that as these individuals had suffered an infectious disease causing death, the remains had been routinely treated to prevent the spread of disease (possibly with formaldehyde), however this could not be confirmed. These remains would not amplify using PCR, and returned very low
endogenous DNA levels. Whether this is due to degradation or some sort of chemical inhibitor is unknown.

Table 11 | Summary statistics for unsuccessful ancient Aboriginal Australian samples

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Location</th>
<th>Sample Type</th>
<th>Age (years)</th>
<th>Endogenous Content (%)</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>aBDV1</td>
<td>Birdsville, QLD</td>
<td>Navicular bone</td>
<td>-</td>
<td>0.07</td>
<td>-</td>
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<tr>
<td>aBDV2</td>
<td>Birdsville, QLD</td>
<td>Tooth</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>DUY1</td>
<td>Duyfken Point, Weipa, QLD</td>
<td>Phalange &amp; tooth</td>
<td>~3,500</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>NORA2</td>
<td>Normanton, QLD</td>
<td>Cranial fragment</td>
<td>-</td>
<td>0.15</td>
<td>Extract turned purple</td>
</tr>
<tr>
<td>NORA3</td>
<td>Normanton, QLD</td>
<td>Cranial fragment</td>
<td>-</td>
<td>0.06</td>
<td>Extract turned purple</td>
</tr>
<tr>
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<td>Cranial fragment</td>
<td>-</td>
<td>0.19</td>
<td>Extract turned purple</td>
</tr>
<tr>
<td>NORA6</td>
<td>Normanton, QLD</td>
<td>Cranial fragment</td>
<td>-</td>
<td>0.23</td>
<td>Extract turned purple</td>
</tr>
<tr>
<td>NORA7</td>
<td>Normanton, QLD</td>
<td>Cranial fragment</td>
<td>-</td>
<td>0.02</td>
<td>Extract turned purple</td>
</tr>
<tr>
<td>PA409.2</td>
<td>Cairns, QLD</td>
<td>Bone</td>
<td>-</td>
<td>N/A</td>
<td>European contamination</td>
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<td>Bone</td>
<td>-</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>WLH3 (LM3 or ‘Mungo Man’)</td>
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<td>Humerus</td>
<td>38,000-42,000 ⁰</td>
<td>N/A</td>
<td>European contamination</td>
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<tr>
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<td>Bone powder</td>
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<tr>
<td>WLH15</td>
<td>Willandra Lakes, NSW</td>
<td>Femur</td>
<td>100 ¹</td>
<td>0.16</td>
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<td>Bone</td>
<td>18,500-22,100 ²</td>
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<td>10,000-17,000 ¹</td>
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<tr>
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<td>-</td>
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<td>WLH28</td>
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<td>100 ²</td>
<td>N/A</td>
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<tr>
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<td>3,000-5,000 ³, 6,000-37,600 ⁴</td>
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<td>-</td>
</tr>
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<td>WLH67</td>
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<td>Femur</td>
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<td>WLH68</td>
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<td>Tibia</td>
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<td>Sample Type</td>
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<td>Endogenous Content (%)</td>
<td>Notes</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>Bone</td>
<td>-</td>
<td>0.35</td>
<td>-</td>
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</tbody>
</table>

**Table key:**

- C = age determined by radiocarbon dating (Gillespie 2002)
- S = age estimate from stratigraphic information (Webb 1989)
- U = age determined by U/Th alpha spectrometry (Gillespie 2002)
- O = age determined by optically stimulated luminescence (Bowler, et al. 2003)

### 2.10 Conclusion

The aim of this chapter was to determine whether it was possible to recover DNA from ancient Aboriginal Australian remains. This DNA could then be used to test whether DNA could assist in facilitating the repatriation of ancestral Aboriginal Australian remains.

Advances in genomic technology in recent years has enabled the successful recovery of nuclear DNA from ancient human remains from tropical, sub-tropical and desert regions such as Ethiopia (Llorente, et al. 2015), Egypt (Schuenemann, et al. 2017), South East Asia (McColl, et al. 2018) and Lebanon (Haber, et al. 2017). A commonality of each of these studies was the coverage of the nuclear genomes recovered, which ranged from 0.01 to 12.5x. This illustrates clearly that while it is possible to recover DNA from ancient samples from such environments it is difficult to reach the levels of coverage often reported from samples found in more temperate climates or permafrost.

It is for this reason, from the outset of this research, it was probable that only a small number of ancient Aboriginal Australian remains would yield usable DNA with which to test this DNA-based repatriation research. While it was not possible to recover aDNA from all sixty ancient Aboriginal Australian individuals, nine nuclear genomes ranging between 0.3 and 6.9x coverage, and thirty mitogenomes ranging between 2.3 and 331.9x coverage were successfully recovered from remains dated between 98 and 1600 years bp.

This demonstrates that while it was impossible in the past using PCR based methods, next-generation sequencing methods has allowed the successful recovery of authentic
aDNA from ancient Aboriginal Australian human remains, despite the climatic conditions. Further, with the future advancement in genomic technologies, this success rate will continue to increase.

The recovery of these genomes represents a major step forward in the recovery of ancient DNA from Australian samples. But more significantly, the recovered ancient nuclear and mitogenomes outlined in this research will contribute considerably to what is currently known about the genomic history of Aboriginal Australians.
Chapter 3: Constructing reference contemporary Aboriginal Australian datasets

Aims of this chapter

The aim of this chapter is to outline the genomic methods used to construct appropriate reference datasets of contemporary Aboriginal Australian genomes with which to compare the DNA recovered from the ancient Aboriginal Australian individuals discussed in Chapter 2. These contemporary genomes will include the 83 nuclear genomes collected and published in Malaspinas et al. (2016) (of which I am a second author) and 18 additional, previously unpublished genomes from three new geographic locations in New South Wales. This chapter will also discuss the different types of DNA that could be used to assist in facilitating the repatriation of ancestral Aboriginal Australian remains, and their respective advantages and disadvantages.

3.1 DNA: background

Human genetic data are a valuable source of information about our past. Our origins, human migration, and patterns of interbreeding have all left evidence within our DNA. Due to the heritable nature of DNA this information remains in the genomes of living and ancient descendants and can provide significant knowledge regarding population history, human evolution and disease (Creanza and Feldman 2016). By identifying the common variants shared across a population and comparing these with those identified in other populations, it is possible to shed light on shared ancestries in the distant past (The 1000 Genomes Project Consortium 2015).

In order to construct a reference genomic dataset of contemporary Aboriginal Australian DNA with which to compare the DNA recovered from ancient Aboriginal Australians, it is important to determine which types of genetic data would be most informative and able to be used effectively with ancient DNA, which is by nature damaged and lower in coverage.

The inheritance of DNA is either uniparental (inherited solely from one parent) or biparental (inherited from both parents). Uniparental DNA is passed from mother to offspring (mitochondrial DNA), or passed from father to son (Y-chromosomal DNA).
Biparentally inherited DNA is passed on in approximately equal proportions from both parents, however is recombined before being inherited (Relethford 2012). Analysis of each of these types of DNA allows researchers to learn about a different aspect of the ancestral past of any given individual.

3.1.1 Characteristics of mitochondrial DNA

While the length of the complete nuclear genome consists of ~3.2 billion base pairs, the circular double-stranded mitogenome measures just ~16.6 thousand base pairs in length (Anderson, et al. 1981; Andrews, et al. 1999). Mitochondrial DNA has been a widely used tool in population genetic studies for over thirty years, since the publication of the first complete human mitogenome in 1981 (Anderson, et al. 1981). Its relative abundance (with hundreds to thousands of copies in each cell) facilitates amplification, even from small amounts of sample template (Pakendorf and Stoneking 2005). Due to its high mutation rate, in non-isolated populations few unrelated individuals will carry the same sequence allowing mutations to be used to trace back ancestral links between populations (Pakendorf and Stoneking 2005). These mutations are not lost to recombination as they are with nuclear DNA, and are inherited as an exact copy making it relatively easy to accurately trace maternal lineages and reconstruct patterns of relatedness (Torroni, et al. 2006). By comparing the mtDNA of populations from around the world it is possible to draw a phylogenetic tree, linking all modern humans to a common maternal ancestor commonly referred to as ‘Mitochondrial Eve’ (Torroni, et al. 2006).

3.1.2 How is mitochondrial DNA variation classified?

The formal classification of different mtDNA types was introduced in the mid-1990s with alphabetical A to G labels assigned to variation observed in Asian and American lineages, H to K for European lineages, and L* used for African lineages (the most ancestral mitochondrial types) in a phylogenetic tree formation all deriving from the most recent common ancestor, ‘Mitochondrial Eve’ (Figure 20) (van Oven and Kayser 2009). Non-African mtDNA lineages are classified into three major groups, or macrohaplogroups, titled ‘M’, ‘N’ and a large subgroup within macrohaplogroup ‘N’ titled ‘R’ (van Holst Pellekaan 2013a). Within these macrohaplogroups are subdivisions
known as haplogroups, some of which are continent or region specific. This is further subdivided to specific haplotypes representing mitochondrial lineages found in smaller groups such as families or local populations.

Each macrohaplogroup, haplogroup and haplotype have specific diagnostic mutations which form the basis of their classification, and by tracing these mutations and analysing their similarities or differences common ancestral links can be inferred.

Figure 20 | Simplified mitochondrial haplogroup classification tree.

The current major macrohaplogroups M, N (and subgroup R), with haplogroups indicated off each branch. Adapted from Phylotree Build 17 (van Oven and Kayser 2009).

3.1.3 Previous contemporary Aboriginal Australian mitochondrial DNA studies

Aboriginal Australians are among the most poorly studied human populations with respect to genetics, with the vast majority of research to date focussed on mitochondrial DNA. Mitochondrial studies of Aboriginal Australians commenced in the late 1990s with much of the early research based on either restriction fragment length polymorphisms (RFLPs) (Huoponen, et al. 2001), or analysis of the coding region of the mitochondrial genome known as the D-loop (HVI) (van Holst Pellekaan, et al. 1998; van Holst Pellekaan, et al. 2006), which represents just 7% of the mitogenome (Ingman, et al. 2000).

All mitogenomic studies of contemporary Aboriginal Australians to date confirm they fall within the M and N macrohaplogroups, specifically in haplogroups O, S, P, R, M, N and Q (van Holst Pellekaan 2013a; Nagle, et al. 2017). It has been previously hypothesised that shortly after Aboriginal Australians colonised Australia, the M and N macrohaplogroup founder types diversified into the Australian-specific haplogroups represented today (van Holst Pellekaan 2013a). Subgroup R (within macrohaplogroup N) is thought to have diversified in Sunda just prior to the colonisation of Sahul (Tabbada, et al. 2010; Delfin, et al. 2014), or in northern Sahul (present day Papua New Guinea) (Gomes, et al. 2015). Then later diversifying into haplotypes unique to both Papua New Guinea and Australia.

Nagle, et al. (2017) recently proposed amendments to the existing Aboriginal Australian mitochondrial DNA classifications due to the identification of novel subclades and haplotypes. These changes will likely be incorporated into a future Phylotree build (van Oven and Kayser 2009). For the purposes of this research we have chosen to manually incorporate these changes into our analyses in order to reflect the latest research findings. While these revisions are covered in detail in Nagle, et al. (2017), a summary of changes made are outlined below (Table 12). Information regarding the variants diagnostic for each of the proposed changes can be found in the Supplementary Tables of Nagle, et al. (2017).
Table 12 | Summary of Aboriginal Australian mitochondrial classifications proposed by Nagle, et al. (2017)

<table>
<thead>
<tr>
<th>Macrohaplogroup</th>
<th>Haplogroup</th>
<th>Sub-Haplogroup</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>M42*</td>
<td>M42a</td>
<td>Subclade M42a1 identified. Further subdivided into M42a1a &amp; M42a1b</td>
</tr>
<tr>
<td>M</td>
<td>M42*</td>
<td>M42c</td>
<td>Novel subclade M42c identified. M42c has at least two subtypes, M42c1 &amp; M42c2</td>
</tr>
<tr>
<td>M</td>
<td>M15</td>
<td>M15</td>
<td>Redefined diagnostic motif</td>
</tr>
<tr>
<td>M</td>
<td>M16</td>
<td>M16</td>
<td>Tentative classification</td>
</tr>
<tr>
<td>M</td>
<td>Q1*</td>
<td>Q1a</td>
<td>No change but indicates potential Torres Strait Island maternal ancestry</td>
</tr>
<tr>
<td>M</td>
<td>Q1*</td>
<td>Q1g</td>
<td>Newly proposed subhaplogroup shared with an individual from the Solomon Islands</td>
</tr>
<tr>
<td>N</td>
<td>N13</td>
<td>N13a</td>
<td>Novel subclade N13a1 identified, &amp; a further subclade N13a1</td>
</tr>
<tr>
<td>N</td>
<td>O1*</td>
<td>O1a1</td>
<td>Novel subclade O1a1 identified</td>
</tr>
<tr>
<td>N</td>
<td>O2</td>
<td>O2</td>
<td>Novel haplogroup O2 identified</td>
</tr>
<tr>
<td>N</td>
<td>S1*</td>
<td>S1b &amp; S1c</td>
<td>Novel subclade S1b identified within S1, &amp; tentatively classified S1c</td>
</tr>
<tr>
<td>N</td>
<td>S2*</td>
<td>S2a &amp; S2b</td>
<td>Novel subclades S2a &amp; S2b identified</td>
</tr>
<tr>
<td>N</td>
<td>S6</td>
<td>S6</td>
<td>Tentative classification</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P4*</td>
<td>P4a &amp; P4b</td>
<td>P4a had been restricted to New Guinea &amp; P4b to Australia. Too few shared mutations for both subtypes to remain within the same P4 clade. P4b has now been reclassified as subclade P11 &amp; P4a drops back to simply P4</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P11*</td>
<td>P11a &amp; P11b</td>
<td>Reclassified P4b to P11, &amp; novel subclades P11a &amp; P11b identified</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P5*</td>
<td>P5a &amp; P5b</td>
<td>Novel subclades P5a &amp; P5b identified</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P6*</td>
<td>P6a &amp; P6b</td>
<td>Novel subclades P6a &amp; P6b identified</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P8</td>
<td>P8</td>
<td>Proposed new basal haplogroup P8 motif, &amp; subtype P8a identified</td>
</tr>
<tr>
<td>N (subgroup R)</td>
<td>P12</td>
<td>P12</td>
<td>Novel clade P12 identified. Further subdivided into P12a &amp; P12b</td>
</tr>
</tbody>
</table>

Table key:

* = asterisk is used to indicate that there are additional subtypes within that haplogroup
3.1.4 Mitochondrial analytical methods

Phylogenetic inferences provide the best estimate of the evolutionary history of a group of individuals, and their relatedness, based on nucleotide similarities and differences contained in the DNA sequences being analysed (Nei and Kumar 2000; Felsenstein 2004; Hall 2011).

A large number of phylogenetics software packages are available (i.e. MEGA, PAUP, PHYLIP, MrBayes) allowing researchers to estimate phylogenies using neighbor-joining, maximum parsimony, UPGMA, Bayesian, distance matrix, and maximum likelihood methods (Nei and Kumar 2000; Felsenstein 2004; Hall 2011; Wiley and Lieberman 2011; Hall 2013). Each of these methods have distinct underlying assumptions, advantages and disadvantages. The maximum likelihood method was first used for nucleotide based research in 1981 (Felsenstein 1981). While other methods, particularly that of maximum parsimony, can fail when assumptions are violated, the maximum likelihood method often produces estimates that have lower variances and is more robust to violations of assumptions (Yang 1994, 1996). This becomes an important consideration when the data being analysed involves moderate to large amounts of change, particularly in circumstances where the number of changes are unequal from lineage to lineage (Felsenstein 1978).

Bootstrapping is often used when constructing phylogenetic trees and is a resampling technique designed to increase the confidence that the final tree is one that has the highest likelihood. A number of iterations are chosen, usually between 100 and 1000, and bootstrapping acts to statistically simulate sub-sampling. Each iteration creates a separate tree and once all iterations are completed, the final phylogenetic tree is produced with bootstrap values for each node and branch (Wiley and Lieberman 2011). The higher the bootstrap value, the more support the particular node or branch is given. For example, a branch with a bootstrap value of 70% (generally accepted as being reasonably reliable) indicates that in 70% of the total iterations the result is what is shown in the final phylogenetic tree (Hall 2013). Bootstrap values below 70% are not necessarily wrong, just not well supported.
A major limitation when using phylogenetic inference is that the final generated tree is only as good as the DNA sequences on which it is based. The resolution of any phylogenetic tree that only utilises the HVS-I or HVS-II region of the mitogenome will be less than a tree which uses complete mitogenomes. Additionally, when a phylogenetic tree is generated using a combination of data, for example using the HVS-I region and complete mitogenomes, the tree will be more informative for the positions in which data exists for all samples, namely that of the HVS-I region.

Other ways in which mtDNA might be analysed is by using methods that investigate the mitochondrial genetic diversity within and between populations such as $F_{ST}$ and analysis of molecular variance (AMOVA). These methods will allow statistical insight into the degree of similarity and difference of the mitogenomes of Aboriginal Australian individuals and populations (Excoffier and Lisher 2010).

### 3.1.5 Characteristics of Y-chromosome DNA

Like the mitochondrial genome, the Y-chromosome (Y-chr) is inherited solely from one parent to another, in this case from father to son. Whereas the mitogenome represents a very small portion of the total length of the human nuclear genome (<0.001%), the Y-chromosome is substantially larger measuring ~60 million bp in length. Approximately 5% of the Y-chromosome is affected by biparental recombination, however the remaining ~95% does not recombine. This portion is referred to as the non-recombining region of the Y-chromosome (NRY, though for ease this portion that will be referred to as Y-chromosome hereafter) (Jobling and Tyler-Smith 1995, 2003). Due to its increased size, the Y-chromosome consists of a much larger range of polymorphisms able to be analysed such as insertions, deletions, inversions and duplications, all of which mutate at different rates (Jobling and Tyler-Smith 1995).

In the same way that mtDNA is informative for investigating maternal population history, analyses of the Y-chromosome can provide insights into the history of paternal lineages. Though, due to its size the Y-chromosome has higher sequence diversity and therefore provides more specificity than mitogenomic analyses (Jobling and Tyler-Smith 1995, 2003).
3.1.6 How is Y-chromosome DNA variation classified?

In the mid-1990s the discovery of numerous Y-chromosome haplotypes enabled researchers to begin building a reference Y-chromosome tree, consisting of many branches all stemming back to the most recent common ancestor, Y-chromosome Adam (Jobling and Tyler-Smith 1995).

The design of the Y-chromosome tree follows the same basic principles as that of mtDNA, with haplogroups being labelled with alphabetical identifiers A-T (Figure 21). However, the geographical differentiation of Y-chromosome haplogroups is even more pronounced, with a far larger number of haplogroups and haplotypes being present.

Many human populations worldwide are traditionally patrilocal in terms of marriage, with men tending to stay close to their place of birth, while women migrate some distance. This can lead to accumulations of distinct Y-chromosome haplotypes in very specific geographic areas (Shen, et al. 2000; The Y Chromosome Consortium 2002).

3.1.7 Previous contemporary Aboriginal Australian Y-chr DNA studies


Through these studies unique Aboriginal Australian Y-chromosome haplogroups, C* and K* predominantly, and M* in rare cases, were identified, and were regarded as likely to have derived from some of the earliest paternal lineages to have left Africa (Nagle, et al. 2015; Bergström, et al. 2016). In previous studies, one constant, however, was the detection of considerable levels of Eurasian admixture, with a large number of research participants self-identifying as Aboriginal Australian carrying non-Indigenous Y-chromosome haplotypes. This level of Eurasian admixture varied from study to study, with ~32% being reported by Malaspinas, et al. (2016), ~56% by Nagle, et al. (2015) and ~70% by Taylor, et al. (2012).

This is in stark contrast to the findings of mtDNA studies. For example, of the 83 complete mitogenomes presented in Malaspinas, et al. (2016) just two individuals (2.4%) carried non-Aboriginal Australian specific mitochondrial haplotypes. High levels of Eurasian Y-chromosome introgression have also been observed in other Pacific populations, with ~27% of Polynesian (Hurles, et al. 1998) and ~40% of self-declared Māori males (Underhill, Passarino, Lin, Marzuki, et al. 2001) having Eurasian Y-chromosome haplotypes. The Eurasian explorers, missionaries and settlers who colonised much of the world, including Australia, over the last 500 years have been predominantly male and as a result many populations exhibit the results of male-biased admixture and directional mating (Jobling, et al. 2013).

When combined with the substantial loss of Aboriginal Australian lives to disease, and the forced separation of Aboriginal Australian males and females into separate missions and reserves, this has further exacerbated the issue. There has undoubtedly been a significant loss of Aboriginal Australian Y-chromosome genetic diversity since European settlement in 1788, perhaps with entire lineages lost to the past. This admixture makes it extremely difficult to obtain a clear picture of the paternal genomic history of Aboriginal Australians and as such, would not be helpful with attempts to find possible ancestral connections for repatriation purposes.
3.1.8 Characteristics of nuclear DNA

When the first human genome was published in 2001 (International Human Genome Sequencing Consortium 2001), the cost of sequencing a complete genome was approximately US $100 million. Since that time sequencing costs have rapidly decreased, with human genome sequencing in 2017 costing approximately US $1000 (Wetterstrand 2017). This has led to an upsurge in the availability of human nuclear data for analyses.

While the analyses of mtDNA is relatively simple for the identification of genealogical histories (Hall 2013), nuclear DNA is more difficult to analyse due to the effects of recombination (Zhang and Hewitt 2003). Through the latter process the genealogical histories stored within each chromosome are lost (Jobling, et al. 2013). The origin of inherited DNA is clear for mitochondrial and Y-chr DNA as there is only one possible ancestor in the previous generation, however it is far more complex with nuclear DNA. With each generation the number of potential ancestors contributing to nuclear DNA doubles (two parents, four grandparents, eight great grandparents and so on) (Relethford 2012).

This makes the reconstruction of genealogical relationships much more complex, but not impossible. Access to an increasing number of nuclear genomes have given rise to new and more intricate population genetic analytical methods being developed. Methods such as Multiple Sequentially Markovian Coalescent (MSMC) analyses have been developed to statistically model ancestral relationships while taking into consideration processes such as recombination and mutational change (Schiffels and Durbin 2014).

One major advantage of this increased amount of data, is that the ~3.2 billion base pairs contained within the human nuclear genome includes a large number of independent loci. This means it is possible to gain a more accurate representation of genetic history than can be obtained through single loci uniparental DNA (Relethford 2012). Analyses such as Principal Component Analysis (PCA) are widely used on SNP data for investigating and summarising population structure and their similarities and differences (Novembre and Stephens 2008). For many of these sorts of analyses, the
target population is compared against one or more of the extensive nuclear reference datasets of worldwide populations available such as the 1000 Genomes Project (The 1000 Genomes Project Consortium 2015) or the Human Genome Diversity Project (HGDP) (Cann, et al. 2002).

Levels of genome-wide admixture can be estimated proportionally using ADMIXTURE software, with reference population data representing ‘ancestral’ populations (Alexander, et al. 2009). While $f3$ outgroup statistics and $D$-statistics can be used to shed light on which populations are descended from each other (Reich, et al. 2009), what genetic contributions were made by one population to another (Green, et al. 2010), or identifying who is the closest relative to a contemporary or ancient population or individual (Raghavan, et al. 2013).

Further analytical methods to test for direct ancestry were developed and used in the study of the ancient remains of the Clovis Anzick Child from Montana in the United States (Rasmussen, et al. 2014) and the famous ancient Kennewick Man (Rasmussen, Sikora, et al. 2015). This likelihood ratio test uses a coalescence model to estimate the branch length of a population tree of two populations, each represented by a single diploid individual (Rasmussen, Sikora, et al. 2015). This method helped finally settle the long and contentious debate regarding Kennewick Man’s ancestral affiliations, and facilitated his return to the appropriate community (Callaway 2016) and his subsequent reburial.

One consideration needed in any genomic research involving contemporary human populations, particularly those who have been at some point in history been colonised by Europeans, is the effect that admixture may have on any analyses. In particular, the question is how to interpret the genomic variation observed when it has been confounded due to admixture with non-Indigenous populations (Kidd, et al. 2012). The ‘noise’ that this genetic input creates can often obscure the small population-level differences that researchers are seeking to investigate (Kidd, et al. 2012). This means that many such sequences must be deconvoluted (have the tracts of local-ancestry identified and assigned) and admixture ‘masked’ (non-indigenous tracts removed) before analyses are completed (Kidd, et al. 2012; Malaspinas, et al. 2016).
3.1.9 Previous contemporary Aboriginal Australian nuclear DNA studies

To date three nuclear DNA studies of contemporary Aboriginal Australians have been published utilising either genome-wide SNPs (McEvoy, et al. 2010; Reich, et al. 2011) or complete nuclear genomes (Malaspinas, et al. 2016).

McEvoy, et al. (2010) analysed 160,337 nuclear SNPs from thirty-eight contemporary Aboriginal Australians from populations from the Riverine region of New South Wales. Their analyses centred on the relationship between their study population, the Paakantji and Ngiyampaa Peoples, and worldwide populations represented in both the HapMap3 (The International HapMap Consortium 2010) and HGDP (Cann, et al. 2002) datasets. They achieved this by constructing a neighbor-joining $F_{ST}$ phylogenetic tree, PCA and analysing levels of admixture. The authors concluded Aboriginal Australians, Melanesians and Papuans formed a distinct clade, supporting the hypothesis that the migration and settlement of Pleistocene Sahul likely occurred through each of these geographic regions. However, the authors acknowledged the necessity for the inclusion of pre-European settlement dated DNA in order to obtain a more accurate picture of the genomic history of Aboriginal Australians, and highlighted the limitations of their research with only a very small geographic region covered (McEvoy, et al. 2010).

Reich, et al. (2011) included contemporary Aboriginal Australian samples in their nuclear SNP-based research aimed at quantifying the levels of Denisovan admixture in populations worldwide. Eight of the ten Aboriginal Australian sequences included in that study originated from an earlier study by Redd and Stoneking (1999) of contemporary Aboriginal Australians from Arnhem Land in the Northern Territory. The remaining two sequences were obtained from a human diversity cell line panel held by the European Collection of Cell Cultures and their origin was not disclosed. The results show Denisovan gene flow occurred into the common ancestors of Papuans, Aboriginal Australians and the Mamanwa (of the Philippines), but not the ancestors of the Jehai (of Malaysia) or the Onge (of the Andaman Islands) (Reich, et al. 2011).

The only research to date that has utilised the complete nuclear genomes of contemporary Aboriginal Australians is that of Malaspinas, et al. (2016). This study focussed on the genomic history of Aboriginal Australians and included 83 complete...
genomes from nine Pama-Nyungan language speaking Aboriginal Australian communities from a wide geographic range. We established that the ancestors of Papuan and Aboriginal Australians differentiated from each other ~25–40kya, and all studied contemporary Aboriginal Australians descend from a single founding population. We inferred a population expansion from northeast Australia in the past 10kyr which correlates strongly with the spread of the Pama-Nyungan language group. We also discovered evidence of archaic admixture within the genomes of contemporary Aboriginal Australians originating from neither Denisovans or Neanderthals, suggesting there are further archaic hominins who contributed DNA to present day populations but are yet to be discovered (Malaspinas, et al. 2016). Whether these results are representative of the non-Pama-Nyungan language speakers of the Northern Territory and the Kimberley region of Western Australia is yet to be determined.

3.2 Constructing reference datasets of contemporary Aboriginal Australian DNA

3.2.1 Identification of appropriate DNA markers and analyses appropriate for assisting with repatriation

The types of analyses possible are very much driven by the type (mtDNA, Y-chromosome or nuclear genomes) and coverage of the aDNA successfully recovered. Nine nuclear genomes ranging between 0.3 and 6.9x coverage, and thirty mitogenomes ranging between 2.3 and 331.9x coverage were successfully recovered from ancient Aboriginal Australian remains. This indicates that both mtDNA and nuclear DNA could be analysed, though the types of nuclear analyses will be limited by some of the lower coverage ancient nuclear genomes.

Mitochondrial analyses will be restricted to phylogenetic inferences for repatriation purposes. Given the size of the Y-chromosome, it is unlikely that many of the ancient Aboriginal Australian individuals will have sufficient coverage of the Y-chromosome to be worthwhile. An additional complication of using Y-chromosome DNA for repatriation is that previous studies of contemporary Aboriginal Australians have shown significantly high levels of European introgression (Taylor, et al. 2012; Nagle, et
al. 2015; Malaspinas, et al. 2016), making the construction of a representative contemporary Y-chromosome dataset extremely difficult, if not impossible.

Nuclear analyses such as $f_3$ and $f_4$ outgroup statistics, Principal Component Analysis (PCA), and direct ancestry likelihood test were successfully used to repatriate Kennewick Man (Rasmussen, Sikora, et al. 2015) and could, therefore, be used to facilitate repatriation of ancient Aboriginal Australian remains. Any contemporary Aboriginal Australian nuclear data included in the repatriation reference dataset would need to first be phased and admixture masked before analysis (Kidd, et al. 2012; Malaspinas, et al. 2016).

### 3.2.2 Contemporary Aboriginal Australian DNA datasets

In order to assess the accuracy of DNA in assisting in the repatriation of ancient Aboriginal Australian remains, a dataset of contemporary Aboriginal Australian mitochondrial and nuclear genomes from which to compare the ancient Aboriginal Australian DNA sequences is required.

One major concern is the availability and suitability of previously published contemporary Aboriginal Australian genomic data. Many of the published contemporary Aboriginal Australian mtDNA studies have focussed on either restriction fragment length polymorphisms (RFLPs) (Huopenen, et al. 2001), or analysis of the coding region of the mitochondrial genome (van Holst Pellekaan, et al. 1998; van Holst Pellekaan, et al. 2006). These sequences, when combined with complete mitogenomes, would limit any phylogenetic inference and would lack the resolution necessary for repatriation. As such, only complete mitogenomes of contemporary Aboriginal Australians were included in the reference dataset assembled.

A further hindrance is that many of these previously published DNA sequences lack accurate and specific geographic information related to their origin. The inclusion of previously published sequences that only specify a state level, for example “Northern Territory”, would not provide the sufficient resolution necessary for assisting with the repatriation issue. As this research sought to have as robust a dataset as possible, researchers were contacted for further information regarding a more precise geographic
location, or language and cultural affiliation for the sequences they had deposited on the National Center for Biotechnology Information (NCBI) genetic sequence database, Genbank, or the European Nucleotide Archive depository. Many researchers did not reply to our requests, while others advised that all the information they were able to provide was stated in their publication.

For example, the recent publication of 111 mitogenomes recovered from historic hair samples from sites in Queensland and South Australia could not be included in the repatriation dataset as the sequences were deposited to the European Nucleotide Archive depository without the geographic identifiers the authors used in their publication (Tobler, et al. 2017). Similarly, the nuclear DNA sequences published by Reich, et al. (2011) of contemporary Aboriginal Australians from the Northern Territory and two sequences from the human diversity cell line panel at the European Collection of Cell Cultures could not be included due to lack of detail.

As such, we constructed the contemporary mitochondrial and nuclear DNA datasets with genetic data we were confident was accurate and precise. This consisted of mitogenomes and nuclear genomes with clearly defined geographic origins and self-identified language affiliations.

3.3 Contemporary Aboriginal Australians: background

The contemporary Aboriginal Australian individuals included in this research are a combination of genomes published in our 2016 Nature publication (Malaspinas, et al. 2016), new unpublished genomes, and previously published and publicly available genomic sequences (Figure 22 and Table 13).

Each of the individuals we approached and consented to participate in this research provided us with background information about their kin, their known ancestry, and believed that they had lengthy connections with the Country in which they currently resided.
All contemporary Aboriginal Australian saliva samples were collected using the Oragene Discover (OGR-500) saliva collection kit (DNAgenotek, Canada). Given Aboriginal Australian feelings about the collection of blood samples for the ‘Vampire Project’, we felt collecting saliva was a far less invasive DNA collection method.

3.3.1 Ethical consent and community permissions

From its inception, this research has been a collaboration between Griffith University and the Centre for GeoGenetics at the University of Copenhagen, together with a number of Aboriginal Australian Traditional Owners and their communities.

Our research began in 2010 when Professor David Lambert initiated a genomic research project in collaboration with the Paakantji, Ngyiampaa and Mutthi Mutthi peoples to
study ancient Aboriginal Australian remains from the Willandra Lakes Region World Heritage Area. In 2011, Professor Eske Willerslev from the Centre for GeoGenetics at the University of Copenhagen commenced discussions with a number of Aboriginal Australian communities including the Wongatha and Ngadju peoples initially intended to gauge support for the publication of the first Aboriginal Australian genome, obtained from a hair sample collected in the Goldfields region in the 1920s (Rasmussen et al. 2011).

After publication, our Aboriginal Australian collaborative partners expressed interest in extending the research to get a broader picture of the genomic history of contemporary Aboriginal Australia by including further Aboriginal Australian communities, particularly those from different Pama-Nyungan language sub-groups. In this regard Tapij Wales played a key role. Research team members from Griffith University collected samples from Eastern Australia (Birdsville, QLD – BDV; Cairns, QLD - CAI and Weipa, QLD - WPA), while the University of Copenhagen collected samples from Western Australia and the Riverine area of New South Wales (Esperance, WA – ENY; Western Central Desert, WA – WCD; Wongatha, WA – WON; Ngadjumaya, WA - NGA and Riverine, NSW - RIV). Each institution obtained its own Human Research Ethics approval and samples were collected under the ethical guidelines set forward by the researcher’s home institution.

Sample collection was planned with the guidance of Aboriginal Australian Traditional Owners from each community. These Traditional Owners, or community appointed representatives, joined the research team in order to initiate contact with Aboriginal Australians interested in participating. In accordance with the National Statement on Ethical Conduct in Human Research (National Health and Medical Research Council 2007), a Human Research Ethics application was submitted (Ref No: ENV/20/13/HREC) with the Griffith University Human Research Ethics Committee (GUHREC). This application included the submission and subsequent approval of the consent package: a plain English information sheet that was provided to all members of the community who were interested in the project, and a consent form (Appendix 7).
Before collecting samples from participants, researchers spoke with the Aboriginal Australian communities and provided printed information booklets to all interested parties. Discussions highlighted the potential benefits and risks of the research, and explained in detail how any recovered genetic information would be treated confidentially and anonymously, using a de-identification system from the time of collection. Signed consent forms are held in trust by a third-party (Dr Don Love, Division Chief, Pathology Genetics at Sidra Medical and Research Center, Qatar), with the research team no longer having access to this information once the sample collection process is completed. As participation was voluntary, participants were advised that they could withdraw from the research at any time. Specific details regarding the collection of saliva samples by researchers from the Centre for GeoGenetics at the University of Copenhagen can be found in Malaspinas et al. (2016).

3.3.2 Malaspinas et al. individuals

A total of 84 saliva samples were collected from nine Aboriginal Australian communities across three states: New South Wales, Queensland and Western Australia (Malaspinas, et al. 2016). While the central aim of the Malaspinas et al. (2016) publication was to characterise the population history of Aboriginal Australia using contemporary DNA, many of the sampling locations were chosen because we had ancient Aboriginal Australian human remains from the same locations and communities. One sample, BDV03, failed sequencing and was excluded from further analyses.

Birdsville, Queensland (BDV01-BDV02, BDV04-BDV10)

Saliva samples were taken from ten Aboriginal Australian people from the Birdsville area, an outback town near the borders of New South Wales, Queensland and South Australia. The participants from this region self-identified as members of the Wangkangurru and/or Yarluyandi language groups.

The Birdsville region has been documented as the centre of extensive trade and cultural networks in traditional times, spanning multiple language groups. One of the most important being the ‘Pituri Track’, a trading network for the pituri plant which was chewed in the days before the arrival of European tobacco (Letnic 2000; Kerwin 2010).
The area encompassing Birdsville and nearby Bedourie was the main growing and processing centres, with Aboriginal Australians travelling very long distances for supplies, some from as far as the West Darling River region of New South Wales and the Western Central Desert in Western Australia (Aiston 1937; Barlow 1979; Kerwin 2010).

European arrival into the area commenced in the 1840s with numerous expeditions passing through, and a cattle industry was established in the 1870s with the Birdsville Track being used to walk cattle from Northern Queensland and the Northern Territory to South Australia (McGinn 1980).

Cairns, Queensland (CAI01-CAI10)

With one exception, the ten participants belong to the Yidindji and Gungandji groups whose Country lies just north and south of the North Queensland town of Cairns.

The Cairns region was settled comparatively later than other regions of Australia. Until the 1850s, the original Aboriginal Australian inhabitants had only occasional contact with non-Aboriginal people (heritage ALLIANCE 2011). From the mid 16th century, commercial harvesting of *bêche-de-mer* (sea cucumbers) began in the region, a process that included the use of local Aboriginal labour by the Macassans from present day Sulawesi (Macknight 1986; McIntosh 1997; Morwood and Hobbs 1997).

It was not until the discovery of gold in the 1870s, and the establishment of the port of Cairns that extensive population exchanges began to occur (heritage ALLIANCE 2011). The goldfields, the port and later the agriculture in the area attracted a variety of outside groups, including Europeans and the Chinese (Kirkman 1993). The Bellenden-Ker, later named the Yarrabah mission, was established as a home for Aboriginal people just south of Cairns in the 1890s. This became the largest mission in Queensland by 1903 (Hume 1991) and people from many different language groups were forced to relocate to Yarrabah (Tindale 1938).

Esperance, Western Australia (ENY01-ENY08)

The eight participants from the Esperance region are Esperance Nyungar, the easternmost subgroup of the large Nyungar language group that occupies the southwest of Western Australia. This is a coastal group with strong ties extending to the west and
weaker links to the east and north (Turney, et al. 2001). To the west of Esperance, the south-western tip of Australia is known to have been occupied at 48ky BP (Turney, et al. 2001).

French and British maritime explorers landed along the south coast of Western Australia in 1792 and 1802, respectively (Dyer 2005; Morgan 2016). In 1826 a European military outpost was set up at Albany on the south coast of Western Australia, approximately 400 kilometres west of Esperance. The township of Esperance was established in 1866 (Gooding 1999).

Ngadjumaya, Western Australia (NGA01-NGA06)
The six participants from Ngadju occupy the country south of Kalgoorlie across to the Southern Ocean at Israelite Bay. Very limited archaeological research has been undertaken in the area. The group has strongest cultural links with Kalamaia-Gubrun to the northwest and the Mirning to the east, with less strong connections to the Nyungar to the southwest and Western Desert groups to the north (Malaspinas, et al. 2016).

Farming was established in the area in 1872 when Fraser Range sheep station was established in the heart of Ngadju country. Gold was discovered in the north-western part of Ngadju country in 1892-1893 and several thousand non-Aboriginal people arrived over the next few years (Malaspinas, et al. 2016).

Pilbara, Western Australia (PIL01-PIL12)
The twelve participants from the Pilbara region belong to the Yinhawangka, Banjima and Guruma groups occupying the Hamersley Range area of Western Australia. European settlement occurred in the 1860s, with farming, gold mining and pearling industries being established (State of Western Australia 2009)

Riverine, New South Wales (RIV01-RIV08)
The eight participants from the Riverine region are all members of or have strong connections to the Paakantji language group, although some identified primarily with the neighbouring Maraura, Ngiyampaa and Kurnu groups. The Paakantji language group occupy an almost 800 kilometre stretch of country along the Darling River from its junction with the Murray River north almost to the Queensland border (Hercus
European arrival into the Riverine region began in 1829, though it wasn’t until the 1840s that cattle stations began being established (Heritage Office and Department of Urban Affairs and Planning 1996).

Western Central Desert, Western Australia (WCD01-WCD13)
The thirteen participants from the Western Central Desert of Western Australia are all from the Ngaanyatjarra language group. Their country extends from just west of the Western Australia/South Australia border in the east, to just north of Kalgoorlie.

Initial occupation of the Western Central Desert arid zone by Aboriginal Australians dates to the Pleistocene and possibly as early as 39ky BP (Smith, et al. 1997). Abandonment of much or perhaps the entire desert zone, with the exception of refugia mainly on the margins, is likely to have occurred during the Last Glacial Maximum (Veth 1989). Permanent settlement of the arid interior resumed or expanded from a small base by the terminal Pleistocene/early Holocene (Smith, et al. 1998). Several European exploration parties visited the Warburton Range area between the 1890s and 1920s but contact was minimal until the 1930s by which time the area was visited regularly by prospectors and would-be pastoralists. In 1934 the Mount Margaret mission opened a branch at Warburton Range, which became an independently operating institution three years later (Green 1983).

Wongatha, Western Australia (WON01-WON11)
Most of the eleven participants from Wongatha were speakers of the Wangkatja dialect, but a minority of individuals belonged to the closely related language groups Tjupan and Koara. These are three most south-western of the Western Desert (Wati) dialect groups. Wongatha country was crossed by numerous overland exploration parties from 1869 until the 1890s. Thousands of non-Aboriginal people began arriving in Wongatha country in 1892 following discoveries of gold at various places north of Kalgoorlie (Malaspinas, et al. 2016). Today, many of the groups’ members live in the towns of Kalgoorlie, Leonora and Laverton in the Goldfields region. They are related to the Western Central Desert participants although each of the groups identify with a particular area of country and have certain restrictions on entering neighbouring areas.
Weipa, Queensland (WPA01-WPA06)

The six participants from this area primarily belong to the Yupangati and/or Thanakwithi language groups, which occupy the country on the western side of Cape York between Albatross Bay and Cullen Point. These groups traditionally relied heavily on marine food resources (Meston 1896). They had extensive contact and trade with people in the Torres Strait (Chase 1981) and Papua New Guinea in the period before European settlement.

Macassan fishermen from Sulawesi visited northern Australia on a regular basis from at least the 16th century until the early twentieth. The Macassans are thought to have travelled regularly east as far as the southern coast of the Gulf of Carpentaria (Russell 2004) and contact with Aboriginal groups on the west coast of Cape York, while not conclusive, is a distinct possibility (Tacon and May 2013).

Church missions were established at Mapoon in 1891 and Weipa in 1898 (Queensland 1899; Wharton 1996; Kidd 1997). Today, most Yupangati and Thanakwithi live in the main regional town of Weipa with some others at Mapoon which remains an Aboriginal community. Samples were taken at those two locations.

3.3.3 Additional samples

An additional 18 saliva samples were collected from individuals from three further locations within New South Wales: Barham, Bourke and Willandra Lakes (see Figure 22 for locations).

Barham, New South Wales (KPM01-KPM04)

The four participants from this area belong to the Barapa Barapa people whose country includes the Perricoota and Koondrook Forests and the townships of Kerang and Barham of the border region of New South Wales and Victoria. Europeans settled in the area in 1843 when the large ‘Barham’ station was established by Edward Green (McConnell 1951). Later the area was settled towards the end of the gold rush by squatters who used the land to graze sheep and cattle (McConnell 1951).
Bourke, New South Wales (BKM01-BKM08)
The eight participants from Bourke in western New South Wales, the traditional home to the Ngemba group of the Wongaibon language group. European explorer Charles Sturt arrived in the area in 1828 during an intense drought and dismissed the area as uninhabitable, and it was not until 1835 that European settlement of the area began with the construction of a military fortification designed to prevent attacks by local Aboriginal Australians (Mitchell 1839).

Willandra/Riverine, New South Wales (WIL01-WIL04, W12 & W26)
Saliva samples were collected from six Aboriginal Australian participants from the World Heritage Willandra Lakes region of far west New South Wales. This area is the traditional meeting place of the Mutthi Mutthi, Nyiampaa and Paakantji Aboriginal tribes.

The Willandra Lakes are an extensive area containing a system of ancient lakes, most of which are fringed by crescent shaped dunes or lunettes. Aboriginal Australians lived on the shores of these lakes for between 47.5 and 55ky BP (Bowler, et al. 2003; Westaway, et al. 2017). Many important archaeological findings have been discovered in the area, most significantly the oldest human remains found in Australia, that of ‘Mungo Man’, and ‘Mungo Lady’, believed to be the oldest site of ritual cremation in the world (43.5kyr BP) (Bowler, et al. 2003). European exploration of the Willandra Lakes region commenced in the 1830s, however it wasn’t until the 1860s that the first real influx of European settlers arrived and established stations for sheep farming (Douglas 2010).

Note: the mitogenomes W12 and W26 were published in Nagle, et al. (2017), however these were not made publicly available, and W26 was further sequenced to obtain a complete nuclear genome.

3.3.4 Previously published sequences
Sixteen previously published Aboriginal Australian mitogenomes were included in this research (van Holst Pellekaan, et al. 2006; Hudjashov, et al. 2007; Rasmussen, et al. 2011) (Table 13). As one of the aims of this research is to determine whether DNA can be used to repatriate unprovenanced ancient Aboriginal Australian remains, only
contemporary sequences that were reliably associated with accurate and specific geographic information relating to their origin were included. This resulted in the exclusion of sequences from many previously published genomic studies of Aboriginal Australians as the geographic locations of the study populations and/or their language groups were not clearly identified (Ingman, et al. 2000; Ingman and Gyllensten 2003; Nagle, et al. 2017; Tobler, et al. 2017), or they were confined to the control region (van Holst Pellekaan, et al. 1998).

Table 13 | Previously published sequences that were included in this study

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<td>W26 (not publicly available)</td>
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<td>Nagle, et al. (2017)</td>
<td>S2a1a</td>
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</table>

3.4 Contemporary genomic methods

For the 83 Aboriginal Australian samples, the details of library construction and HiSeq sequencing can be found in the Methods section of Malaspinas, et al. (2016). For the 18 new Aboriginal Australian samples, DNA library construction and sequencing were completed by the Kinghorn Centre for Clinical Genomics (KCCG) at the Garvan
Institute in Sydney, Australia or our collaborative partners, Novogene Bioinformatics Technology Corporation Limited in Beijing, China. Sequencing libraries were generated using Truseq Nano DNA HT Sample Preparation Kit (Illumina) following the manufacturer’s recommendations. Libraries were then 150 base pair paired-end sequenced on an Illumina HiSeq X. Sequences were base-called using CASAVA 1.8.2 (Illumina), FASTQ file generated and demultiplexed by the sequencing facility. Coverage of contemporary sequencing averaged between 45-60x.

3.5 Genomic data processing

3.5.1 Contemporary data filtering and processing
Paired-end DNA sequences were mapped to the human reference genome (GRCh37/hg19) or the revised Cambridge reference mitochondrial genome (Andrews, et al. 1999) using BWA 0.6.2 (Li and Durbin 2010). Sequence Alignment/Map (SAM) files were then further processed using SAMtools (Li, et al. 2009), removing duplicates. Using the SAMtools mpileup command, with a maximum depth parameter of 1000, variant call format (VCF) files were generated for each chromosome separately. Using an awk command indel variations were excluded. The VCF files of individual contemporary genomes were merged using VCFtools (Danecek et al., 2011) after zipping and indexing using tabix (Li 2011b).

3.5.2 Mitochondrial haplotype determination
In some cases, the consensus sequence generated contained ambiguous DNA bases (indicated by the letters Y or R, rather than A, C, G or T). These ambiguous bases can occur due to sequencing errors or miscalled bases during bioinformatic processing, or may indicate the presence of exogenous contamination. In these instances, each base was checked and manually corrected in the consensus sequence. These calls were made solely for bases with a minimum of 3x coverage with the consensus base being present in more than 50% of the reads. In instances not meeting these minimum requirements the base was marked as having no cover, indicated by an N.

Mitochondrial haplotypes were determined using HaploGrep 2.0 software (Weissensteiner, et al. 2016), with mitochondrial variations described in PhyloTree.
mtDNA build 17 (van Oven and Kayser, 2009) or manually using new Aboriginal Australian mitochondrial haplogroup classifications proposed by Nagle, et al. (2017). In cases in which novel haplotypes were discovered we were assisted with the classifications by Mannis Van Oven from Erasmus MC University Medical Centre in Rotterdam.

3.6 Bioinformatic analyses

3.6.1 Mitochondrial phylogenetic analysis

All mitogenomes were aligned using MEGA7 software (Kumar, et al. 2016) software using the MUSCLE parameter (Edgar 2004). The alignment output was checked manually and adjusted where necessary using SeaView (4.6.1), a graphical user interface for multiple sequence alignment and molecular phylogeny (Gouy et. al., 2010).

To obtain the best substitution model the ‘Find Best DNA/Protein Models (ML)’ function was used. Once the best model was identified. Maximum Likelihood phylogenetic trees based on the Tamura-Nei model (Tamura and Nei 1993) were generated using MEGA7 (Kumar, et al. 2016) software, and using 1000 iterations for bootstrap scores. The tree with the highest log likelihood was used. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (categories (+G, parameter =)). This rate variation model allowed for some sites to be evolutionarily invariable ([+I], % sites). The heuristic model used for branch swapping was the Nearest-Neighbor Interchange (NNI).

All positions containing gaps and missing data were eliminated. Subsequent annotation and colouring of the tree were conducted using the Interactive Tree of Life (iTOL) 3.4.3 software (Letunic and Bork 2016).
3.6.2 Nuclear analysis panel

Genotyping for newly sequenced as well as previously reported contemporary individuals was carried out using SAMtools/bcftools (Li and Durbin 2010), followed by filtering as previously described in (Sikora, et al. 2017). For population genetic analyses, those genotypes were merged with a reference panel of 2,286 contemporary individuals from worldwide populations, genotyped at 593,610 SNPs using the Affymetrix HumanOrigins array (Lazaridis, et al. 2014; Lazaridis, et al. 2016). The ancient individuals were processed similarly, represented by pseudo-haploid genotypes obtained by sampling a random allele at each SNP position using the method outlined in Skoglund, et al. (2012) and Green, et al. (2010). Additional diploid genotyping was carried out for the two ancient Aboriginal Australian individuals with the highest nuclear coverage, KP1 (6.9X) and MH8 (6.8X).

3.6.3 ADMIXTURE analyses

ADMIIXTURE v.1.23 software (Alexander, et al. 2009) was used to perform model-based clustering to investigate the patterns of recent admixture. Using supervised mode, five populations were used as putative source populations. This included African (Yoruban), European (French), East Asian (Han), Oceanian (Papuan) and Australian (WCD) individuals. The least unadmixed Western Central Desert Aboriginal Australians were used as representatives of the Australian component.

Specifics regarding all other nuclear data processing are covered in detail in Chapter 5.

3.7 Mitochondrial DNA dataset preparation

The mitochondrial haplotypes of 99 contemporary Aboriginal Australians (excluding previously published sequences) were determined using HaploGrep 2.0 (Weissensteiner, et al. 2016) or manually assigned using the reclassifications proposed by Nagle, et al. (2017) (Table 14).

Table 14 | Contemporary mitochondrial haplogroups and haplotypes observed

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</tr>
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</tr>
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<td>P11a1</td>
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</tr>
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<td>Willandra, NSW</td>
<td>K2a3</td>
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### 3.7.1 Non-exclusive Aboriginal Australian mitochondrial haplotypes

Of these 99 contemporary Aboriginal Australia mitogenomes, five individuals (5.05%) carried maternal haplotypes not exclusive to Aboriginal Australians, representing European and Southeast Asian maternal ancestry (Table 15). These samples were excluded from the dataset.

#### Table 15 | Samples with non-Aboriginal Australian maternal ancestry

<table>
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<th>Code</th>
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<th>Haplotype</th>
<th>Origin</th>
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<tbody>
<tr>
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<td>Bourke, NSW</td>
<td>H5</td>
<td>Europe (University of Otago 2014)</td>
</tr>
<tr>
<td>CAI10</td>
<td>Cairns, QLD</td>
<td>H1e1a</td>
<td>Europe, particularly Portugal &amp; Spain (University of Otago 2014)</td>
</tr>
<tr>
<td>ENY01</td>
<td>Esperance, WA</td>
<td>E1a1b</td>
<td>Island Southeast Asia (University of Otago 2014)</td>
</tr>
<tr>
<td>RIV03</td>
<td>Riverine, NSW</td>
<td>H1b5</td>
<td>Eastern Europe, North &amp; Central Asia (University of Otago 2014)</td>
</tr>
<tr>
<td>WIL03</td>
<td>Willandra, NSW</td>
<td>K2a3</td>
<td>Northern and Eastern Europe (University of Otago 2014)</td>
</tr>
</tbody>
</table>

The remaining 94 individuals were included in a phylogenetic analysis using MEGA7 software (Kumar et al., 2016). To obtain the best substitution model the ‘Find Best DNA/Protein Models (ML)’ function was used. This analysis suggested the Tamura-Nei + Gamma model was best, and was then used to construct an unrooted Maximum Likelihood tree using with 1000 bootstrap iterations. An unrooted tree was chosen as the primary interest was the relationships between the Aboriginal Australians, rather than evolutionary change.
The tree with the highest log likelihood (-27728.3806) is shown (Figure 23). A discrete Gamma distribution was used to model evolutionary rate differences among sites (categories +G, parameter =). This rate variation model allowed for some sites to be evolutionarily invariable ([+I], % sites). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The heuristic model used for branch swapping was the Nearest-Neighbor Interchange (NNI).

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 94 nucleotide sequences and all positions containing gaps or missing data were eliminated. A total of 16,565 positions were used in the final tree. The phylogenetic tree was annotated and coloured using the Interactive Tree of Life (iTOl) v3.4.3 software (Letnic and Bork, 2016).
Figure 23 | Maximum likelihood phylogenetic analysis of contemporary Aboriginal Australians

Each coloured and annotated section represents individual mitochondrial haplotypes.
3.7.2 Novel Aboriginal Australian mitochondrial haplotypes

After constructing the maximum likelihood phylogenetic tree it became clear a number of individuals fell outside the previously published mitochondrial haplotypes of contemporary Aboriginal Australians (van Holst Pellekaan 2013b; Nagle, et al. 2017).


Malaspinas, et al. (2016) was the first study to include populations from the south-western regions of Western Australia. Site frequency spectrum (SFS) analyses showed that the populations of the south-western region of Western Australian and north-eastern regions of Queensland had diverged from one another as early as ~31kya (95% CI 10-32kya), followed by limited gene flow. It was hypothesised that the aridification of the Australian interior during the Last Glacial Maximum acted as a barrier to migration, isolating the populations for long periods of time (Malaspinas, et al. 2016). The distinct regional differentiation observed in the maximum likelihood phylogenetic analyses generated (Figure 29), particularly involving Western Australian populations, supports this hypothesis. The Aboriginal Australian populations of Western Australia carry very different mitochondrial haplotypes to those from the eastern Australian states of Queensland and New South Wales. This was not covered in very much detail in Malaspinas et al. (2016) and is further investigated here.

In order to classify these potentially novel findings accurately, I consulted with Mannis Van Oven, author of PhyloTree (van Oven and Kayser 2009) from the Erasmus MC University Medical Centre in Rotterdam. With his guidance, a number of new subclades were identified, and amendments to existing Aboriginal Australian mitochondrial haplogroup classifications were proposed. These are outlined below:

M42c1b and M42c1b1

Twelve Aboriginal Australian individuals from Western Australia (two from Esperance, five from the Western Central Desert and five from the Pilbara) were found to form two
novel subclades of the newly classified M42c* haplogroup proposed by Nagle, et al. (2017) (Figure 24).

![Figure 24](image)

**Figure 24 | Novel mitochondrial clades M42c1b and M42c1b1**

All sequences listed are from this research.

R12a, R12a1 and R12b

Ten Aboriginal Australian individuals from Western Australia (three from Wongatha, one from Esperance, one from Nyungar, three from the Western Central Desert and two from the Pilbara) were found to form two subclades of the haplogroup R12*. Previously an Aboriginal Australian from the Northern Territory (GenBank accession number DQ1125752) (Kivisild, et al. 2006) had been the sole representative of the R12 haplogroup.

The Western Australian mitochondrial sequences from this research have led to haplogroup R12 and R21 being decoupled from one other. R21 have been found in Malaysian Negritos (Macaulay, et al. 2005), in Sumatra (Gunnarsdóttir, et al. 2011), and in Borneo (Kusuma, et al. 2015). On Phylotree build17 (van Oven and Kayser 2009) haplogroups R12 and R21 share three mutation at nucleotide positions 10398, 11404 and 16295. Given these new R12* mitochondrial sequences, haplogroup R12 will now be redefined with new diagnostic mutations, and subtypes R12a, R12a1 and R12b have been created (Figure 25).
3.7.3 Aboriginal Australian mitochondrial dataset

The final contemporary Aboriginal Australian mitochondrial dataset consists of 112 Aboriginal Australian individuals from four states and territories across Australia: New South Wales, Northern Territory, Queensland and Western Australia. This reference dataset includes 94 contemporary Aboriginal Australians who volunteered to provide samples for this research (80 individuals from Malaspinas, et al. (2016) and 14 additional unpublished samples). To this, 18 previously published complete mitogenomes of known geographic location were included (van Holst Pellekaan, et al. 2006; Hudjashov, et al. 2007; Rasmussen, et al. 2011; Nagle, et al. 2017) (Table 13).

These mitogenomes include representatives of each of the mitochondrial haplogroups and haplotypes within the previously established mitochondrial genetic diversity of contemporary Aboriginal Australians, and as such represent a strong comparative dataset from which to develop a test for the identification of unprovenanced ancient remains.
3.8 Nuclear DNA dataset preparation

Any nuclear DNA reference dataset constructed must take into consideration the effects of admixture, particularly if the study population has become admixed through genetic introgression by non-Indigenous populations. It has been well-established from previous analyses of contemporary Aboriginal Australian Y-chromosomes that 32 - 70% of Aboriginal Australian males carry non-Aboriginal specific Y-chromosome haplotypes (Taylor, et al. 2012; Nagle, et al. 2015; Malaspinas, et al. 2016). As the nuclear genome is recombined during meiosis, incorporating segments from both the maternal and paternal chromosomes (Zhang and Hewitt 2003), it would be fair to assume based on the results of these previous studies, that many of our 100 contemporary Aboriginal Australians likely carry considerable levels of admixture. Before these nuclear genomes can be used the level of this admixture needs to be established, and then deconvoluted and ‘masked’ before analysis. Without this, admixture can confound the accuracy of any analyses performed (Kidd, et al. 2012; Malaspinas, et al. 2016).

The levels of admixture present in the contemporary Aboriginal Australians included in this research was estimated using reference population data to represent putative source populations. Given what we know about European settlement of Australia the putative source populations were European, East Asian and Oceanian. To separate out the ‘Australian’ component the least admixed Aboriginal Australian population was used as representatives, that of the Western Central Desert in central Western Australia (WCD).

Figure 26 | Supervised admixture of contemporary Aboriginal Australians using five putative ancestry sources
Where maroon is African (Yoruban), orange is European (French), dark blue is East Asian (Han), light blue is Oceanian (Papuan) and yellow is Australian (WCD).

What is clear from the resulting ADMIXTURE plot is that the contemporary Aboriginal Australian individuals display varying levels of admixture, depending on their geographic location.

Some contemporary Aboriginal Australian individuals of Cairns (CAI) and Weipa (WPA) in Queensland appear to carry substantial segments of Oceanic and East Asian admixture, which is consistent with what might be expected based on their geographic proximity to Papua New Guinea and the Torres Strait. Aboriginal Australian individuals from populations such as Bourke (BKM), the Riverine (RIV) and Willandra Lakes (WIL) in New South Wales were the most admixed, carrying substantial tracts of European admixture.

These results show it is necessary to ‘mask’ this admixture, before these 100 contemporary nuclear genomes can be used to facilitate the repatriation of ancient Aboriginal Australian remains. How this was done, and the results of these analyses will be covered further in Chapter 5 of this thesis.

3.8 Conclusion

The aim of this chapter was to determine the DNA markers that could reliably be used to construct appropriate reference datasets of contemporary Aboriginal Australian genomic variation. Using these datasets, we can compare the genomic variation of contemporary individuals with that recovered from the ancient Aboriginal Australia individuals discussed in Chapter 2. These datasets will then potentially facilitate the repatriation of ancestral Aboriginal Australian remains.

Through the investigation of both previously published research and new analyses presented here, it was clear, based on the high levels of European Y-chromosome haplotypes being present in contemporary Aboriginal Australian males, that these sequences would not be reliable for the accurate repatriation of ancestral remains. These were therefore excluded from later consideration.
In contrast, an overwhelmingly positive result was achieved through the analyses of mitochondrial DNA genetic diversity amongst the contemporary Aboriginal Australians included in this research with only 5.05% of participants carrying non-exclusively Aboriginal Australian mitochondrial haplotypes. Combined with the high levels of coverage of the mitogenomes of the ancient Aboriginal Australian individuals, these 112 contemporary Aboriginal Australian individuals represent a strong comparative dataset from which to test whether DNA can be used to assist in the repatriation of unprovenanced ancient remains.

Additionally, a nuclear DNA dataset of 100 contemporary Aboriginal Australians, comprising both previously published and newly collected genomes, will also be used. However, *ADMIXTURE* analyses show varying levels of admixture present among Aboriginal Australian populations based on their geographic location. Populations in geographic locations closer to areas that were central to early European settlement clearly show higher levels of European admixture, as would be expected. As such, the effects that this admixture might have on downstream analyses will need to be considered before these analyses are undertaken.

Most significantly, through this process of establishing reference datasets with newly presented contemporary Aboriginal Australian mitochondrial and nuclear genomes, new mitochondrial discoveries were made. Novel mitochondrial haplotypes were observed in a number of individuals from Western Australia. Two novel subclades of the newly classified M42c* haplogroup were discovered, M42c1b and M42c1b1. Additionally, novel R12* haplotypes resulted in the restructure of that particular mitochondrial lineage, with the R12 and R21 haplotypes being decoupled, and the creation of novel subtypes R12a, R12a1 and R12b.

These contemporary reference datasets represent a strong base with which to compare the ancient genomic sequences recovered in Chapter 2. Given the complexities involved in terms of genomic admixture from non-Indigenous populations since settlement of Australia, mitochondrial and nuclear DNA are the best options for testing whether the proposed repatriation method works.
Chapter 4: Can mitochondrial DNA assist with repatriation?

4.1 Aim of the chapter

The aim of this chapter is to directly test whether the reference ‘mitogenomic map’ of contemporary Aboriginal Australian genomic variation that was constructed, when compared with the mitogenomes recovered from ancient Aboriginal Australian remains, can facilitate their repatriation to Country. In order to assess the accuracy of the results generated, a set of comparative benchmarks will be evaluated and the benefits and limitations of each option discussed. Using these benchmarks, it will then be possible to determine whether mitochondrial genomic variation can be used to establish the origin of ancestral human remains, while making observations regarding the mitochondrial diversity of ancient and contemporary Aboriginal Australians.

4.2 Constructing comparative benchmarks for assessing accuracy

Chapters 2 and 3 of this thesis have shown it is possible to successfully recover both mitochondrial and nuclear genomes from ancient Aboriginal Australian remains. Reference ‘maps’ of contemporary Aboriginal Australian genomic variation (both mitochondrial and nuclear) were constructed, allowing us to directly test whether the origin of ancient Aboriginal Australian remains can be established using DNA-based methods. These analyses were conducted using ancient Aboriginal Australian remains from known locations as proxies for unprovenanced remains. If such a comparative test of mitogenomic variation can provide accurate answers, this tool could assist in facilitating the repatriation of ancestral Aboriginal Australian remains from museum collections worldwide.

Given the significance of this endeavour, for Aboriginal Australians, as well as for science, it is imperative that the accuracy of any results generated are evaluated using a set of comparative benchmarks. The first method we evaluated used geographic distance, comparing the current location of contemporary Aboriginal Australians with the location from which the series of ancient Aboriginal Australian remains were recovered. This method had the potential to be complicated by a history of forced relocation and/or voluntary movement in the years following European settlement.
Using geographic distance or geographic location as a benchmark is also problematic as it involves quantifying acceptable and unacceptable distances arbitrarily. Are distances of 50 km, 100 km or 1000 km between ancient and contemporary Aboriginal Australian individuals close enough? As this would be, by its nature, subjective, it is unlikely that this method alone would provide the level of accuracy repatriation requires.

Therefore, a combination of language group affiliations and geographic distance was evaluated. During DNA sampling visits, demographic information was obtained from each contemporary Aboriginal Australian participant regarding the language group with which they self-identified. These language affiliations are often passed from parent to offspring, resulting in many contemporary individuals self-identifying with more than one group. In circumstances where participants did not know their language affiliations, perhaps due to fractured histories or the effects of the ‘Stolen Generation’, the language group present in the region in which they currently resided was assigned.

Both of the above comparative methods involved matching ancient Aboriginal Australians genetically to one or more contemporary Aboriginal Australians. However, in reality repatriation and discussions regarding the eventual fate of ancestral Aboriginal Australian remains would most certainly not occur amongst individuals, but at a community level. As we wished to respect existing Aboriginal Australian societal systems and processes, we sought a method/approach in which to identify suitable communities.

Many Aboriginal Australian communities have formal governance systems in place in the form of Indigenous Corporations and Local Aboriginal Land Councils (LALCs), whose primary role is to represent Aboriginal Australian communities on matters involving land rights, social and economic improvement, and protection of cultural heritage (The Australian Indigenous Governance Institute n.d.). However, the definition of ‘community’ within Aboriginal Australian society can vary considerably (Hunt 2013). Some are distinct geographic settlements; while others are defined by ‘cultural boundaries’ associated with traditional networks or language. These cultural boundaries are often not defined in the same way that the perimeters of a town, city or state are typically indicated (Hunt and Smith 2006; Hunt 2013). As such, we investigated using
publicly available material, such as Aboriginal Australian language and culture maps, to assist us.

Aboriginal Australian language groups are broadly classified into two families: Pama-Nyungan and non-Pama-Nyungan, with approximately 90% of Aboriginal Australian languages belonging to the Pama-Nyungan family geographically (Figure 27).

Figure 27 | Map of the distribution of the Pama-Nyungan and Non-Pama-Nyungan Aboriginal Australian language groups

Major Non-Pama-Nyungan language groups are highlighted in purple or mustard. The distribution of the Pama-Nyungan language family is highlighted in beige (Wright and Lambert 2015).

While it is generally accepted that there are more than 25 Pama-Nyungan language sub-groups, there has been considerable debate amongst linguists regarding both the classification of individual Pama-Nyungan language sub-families and how these sub-families are related to one another (Bowern and Atkinson 2012).

Two specific language maps could be used to assess and categorise our genomic results. The first, the Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS) map of Indigenous Australia (Horton 1996), is highly detailed and displays a large number of language, tribal and nation groups. Before we could use this map we were required to seek permission from AIATSIS, the copyright holders of the map.
However, on application our request was declined. The reason for this was the map reflects data collected between 1988 and 1994, and excludes smaller groups such as clans, dialects or individual languages. The map has also been heavily contested by a number of Traditional Owners who felt the map is incorrect. Given these issues, AIATSIS advised their map was not precise enough to meet our goals and stated it was unsuitable for our research (Australian Institute of Aboriginal and Torres Strait Islander Studies, personal communication, 16-25 August, 2017).

The alternative was an Aboriginal Australian language map published by Bowern and Atkinson (2012), showing the commonly agreed upon major sub-groupings of the Pama-Nyungan language group (Figure 28). While this map is not as detailed as the AIATSIS map, it contained all of the major sub-groups and would enable us to allocate a target language group for ancient Aboriginal Australian remains, although not in the detail we would have liked.

![Figure 28 | Map of the major sub-groups of the Pama-Nyungan language group](image)

(Bowern and Atkinson 2012)

Using the classifications outlined in Bowern and Atkinson (2012) we assigned major language sub-groups to each contemporary Aboriginal Australian based on the language information we were provided or current geographic location (Table 16).
<table>
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<tr>
<th>Sample ID</th>
<th>Location</th>
<th>Language *</th>
<th>Major Language Sub-groups</th>
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</thead>
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<td>Yarluyandi &amp; Wangkangurru</td>
<td>Karnic</td>
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<td>Thaynakwith &amp; Linngithig</td>
<td>Paman</td>
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<td>Mpakwithi &amp; Kaanju</td>
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<td>Mabuiag &amp; Thaynakwith</td>
<td>WTS &amp; Paman</td>
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<td>Mpakwithi</td>
<td>Paman</td>
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</tbody>
</table>

* Language group with which the speaker self-identifies, or to which they were assigned. Where more than one language is given, speakers either identified with more than one group, or they could not be assigned to a single group with certainty.

4.3 Mitochondrial phylogenetics of ancient and contemporary Aboriginal Australians

A total of 143 mitogenomes, consisting of 31 ancient (30 from this research and ANC, the hair sample from Rasmussen et al. (2011)) and 112 contemporary Aboriginal Australians) were used to construct a maximum likelihood phylogenetic tree with MEGA7 software (Kumar et al., 2016). To obtain the best substitution model the ‘Find Best DNA/Protein Models (ML)’ function was used. This analysis suggested the Tamura-Nei + Gamma model was best, and was then used to construct a Maximum Likelihood
tree using with 1000 bootstrap iterations (Figure 29). An unrooted tree was chosen as the primary interest was the relationships between the Aboriginal Australians, rather than evolutionary change.

A discrete Gamma distribution was used to model evolutionary rate differences among sites (categories +G, parameter =). This rate variation model allowed for some sites to be evolutionarily invariable ([+I], % sites). Initial trees were produced by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, before the topology with a superior log likelihood value (-19648.2315) was selected. The heuristic model used for branch swapping was the Nearest-Neighbor Interchange (NNI).

The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A total of 11,042 nucleotide positions were used in the final phylogenetic tree, with positions containing gaps or missing data eliminated. The phylogenetic tree was annotated and coloured using the Interactive Tree of Life (iTOL) v3.4.3 software (Letnic and Bork, 2016).
Each coloured and annotated section represents individual mitochondrial haplotypes. Ancient Aboriginal Australian individuals appear in bold text.

4.3.1 Ancient Aboriginal Australian mitochondrial haplotypes

The haplotypes of all recovered ancient Aboriginal Australian mitogenomes fit within the previously published major classifications of Aboriginal Australian mtDNA. This includes macrohaplogroup ‘M’ (Q* and M*), macrohaplogroup ‘N’ (O* and S*) and the ‘R’ subgroup of ‘N’ (P* and R*) (van Holst Pellekaan 2013b) (Table 17).
Table 17 | Details of ancient Aboriginal Australian mitochondrial haplotypes and assigned major language sub-group

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Location</th>
<th>Haplotype</th>
<th>Major language sub-group of location where remains were recovered</th>
</tr>
</thead>
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<td>Paakantji</td>
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<td>Ancient FLI2</td>
<td>Flinders Island, QLD</td>
<td>P5b1</td>
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<tr>
<td>Ancient KP1</td>
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<td>S2a1a</td>
<td>Kulin</td>
</tr>
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<td>Ancient KP2</td>
<td>Barham, NSW</td>
<td>S2a1a</td>
<td>Kulin</td>
</tr>
<tr>
<td>Ancient MH1</td>
<td>Mapoon, QLD</td>
<td>P5a1a</td>
<td>Paman</td>
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<td>Paman</td>
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<tr>
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<td>Paman</td>
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<td>Ancient MH4</td>
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<td>P12a1</td>
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</table>

4.3.2 Novel ancient Aboriginal Australian mitochondrial haplotypes

A number of ancient mitogenomes fell outside the range of the previously published mitochondrial haplotypes of Aboriginal Australians (van Holst Pellekaan 2013b; Nagle, et al. 2017). Mannis Van Oven assisted with identifying a number of new subclades and
proposing amendments to existing Aboriginal Australian mitochondrial haplogroup classifications. Details of these haplotypes are outlined below:

**M42a3**

Kaakutja (BK1) from Bourke, New South Wales shares several mutations with a contemporary Aboriginal Australian individual (DQ404442.3 from the Riverine) (van Holst Pellekaan, et al. 2006). However, Kaakutja carries a mutation at position 6104T that DQ404442.3 lacks (Figure 29). This 6104T mutation now becomes one of the defining motifs (the mutations specific to a haplotype) of M42a*. This resulted in all sub-haplogroups below being relabelled, with M42a1a becoming M42a1; M42a1b becoming M42a2, while Kaakutja (BK1) and DQ404442.3 formed a novel subclade labelled M42a3.

**Figure 30 | Novel mitochondrial halotype M42a3**


**P11a1**

The mitogenome of NORA4 from Normanton, Queensland resulted in mutation 6881G being removed from the motif of subtype P11a1 (Figure 31). NORA4 represents a new subtype within P11a1. Figure 31 shows the diagnostic mutations carried by each subtype and the sample IDs of the individuals who carry those types.
### Figure 31 | Mitochondrial haplotypes P11a and P11a1


#### P5b1

FLI2 from Flinders Island, Queensland represented a new mitochondrial subtype within the newly created P5b1 haplotype (Figure 32). Figure 32 shows the diagnostic mutations carried by each subtype and the sample IDs of the individuals who carry those types.

### Figure 32 | Novel mitochondrial haplotype P5b1


#### P5a1a and P5a1a1

MH1, MH6 and MH8 from Mapoon, Queensland belong within the newly created P5a* haplogroup, carrying a P5a1a haplotype (Figure 33). Figure 33 shows the diagnostic mutations carried by each subtype and the sample IDs of the individuals who carry those types.
MH4 and MH7 from Mapoon, Queensland were added to the newly created P12a1 haplogroup, with nucleotide position 326G added to the motif (Figure 34). Figure 34 shows the diagnostic mutations carried by each subtype and the sample IDs of the individuals who carry those types.
P12b

The mitogenome of PA86 from Cairns, Queensland was added to the newly created P12b haplotype (Figure 35). Figure 35 shows the diagnostic mutations carried by each subtype and the sample IDs of the individuals who carry those types.

Figure 35 | Mitochondrial haplotype P12b

PA86 is from this study, all other sequences (Nagle, et al. 2017)

4.4 Mitochondrial genetic diversity of ancient & contemporary Aboriginal Australians

Though genetic research of Aboriginal Australians has been limited, what is known from previous mitochondrial studies is that the haplogroups carried are of deep antiquity and highly distinct (van Holst Pellekaan 2013a; van Holst Pellekaan 2013b; Nagle, et al. 2017). Estimates of the time to most recent common ancestor (TMRCA) for Aboriginal Australian mitochondrial haplogroups have been presented in a number of previously published studies, calculated using molecular evolutionary mutation rates estimated from differing methods (Hudjashov, et al. 2007; Soares, et al. 2009; Behar, et al. 2012; Fu, et al. 2013; van Holst Pellekaan 2013a; Nagle, et al. 2017; Tobler, et al. 2017). While there are some differences in these estimates, the median TMRCA of major basal haplogroups (M42a, M42c, N13, O, S and P) date to > 40kya (Nagle, et al. 2016; Nagle, et al. 2017), making them some of the most ancient mitochondrial lineages outside of Africa.

Previously published studies have reported high levels of genetic diversity, of both mitochondrial and nuclear DNA, across the Australian continent (Malaspinas, et al.
2016; Nagle, et al. 2016; Nagle, et al. 2017). Through analyses of the mitochondrial DNA recovered from 143 ancient and contemporary Aboriginal Australians from communities from New South Wales, Northern Territory, Queensland and Western Australia, a similarly high level of mitochondrial genetic diversity was observed. Among the 143 mitochondrial sequences recovered, 102 distinct mitochondrial lineages were observed which were then classified into 38 previously recognised or novel haplotypes. Haplotype diversity (Hd) was calculated using DNA sequence polymorphism software, DnaSP (version 6.10.04) (Rozas, et al. 2017). A value of Hd = 0.9933 ± 0.0013 was obtained, indicating a high diversity of unique haplotypes exists among the Aboriginal Australian individuals included in this research. This is illustrated in Table 18, which outlines the haplotype frequencies within each of the Aboriginal Australian communities sampled. Even in communities where there were only a small number of individuals, a large number of different haplotypes were observed.

It is important to note possible biases resulting from the inclusion of previously published sequence data. Such sequences were included in this research if they had specific geographic information associated with them. However, it is unknown whether these sequences were selected for publication in order to illustrate the presence of particular haplotypes, or whether they were randomly sampled from the communities they originated from. We can affirm that any sampling undertaken for this research was random and great lengths were taken to avoid collecting from contemporary Aboriginal Australians who believed themselves related to other participants. However, given the turbulent history and the remoteness of some of our sampling locations, it was impossible to be absolutely sure if people were related and if so, how close this relationship was at the time of sample collection.

Table 19 outlines the total number of ancient and contemporary Aboriginal Australians carrying specific mitochondrial haplotypes, and the percentage appearing in each state or territory. Many of the haplotypes observed appear to be geographically localised, with a very defined western versus eastern Australia split, or even to specific regional areas such as the Riverine or Cape York Peninsula, while others were much more widespread.
Overall state and territory based mitochondrial haplotype distributions can be seen in Figure 36.
Table 18 | Mitochondrial haplotype distributions

(A) indicates ancient individuals

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Barham NSW (n = 6)</th>
<th>Birdsville QLD (n = 9)</th>
<th>Bourke NSW (n = 8)</th>
<th>Cairns QLD (n = 13)</th>
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4.4.1 Lineages within macrohaplogroup M

Fifteen mitochondrial haplotypes (39.5% of the total haplotypes observed) belonged to macrohaplogroup M. A total of 39 ancient and contemporary Aboriginal Australians carried these mitochondrial lineages (27.3%).

Mitochondrial haplotypes M14 and M15 have been previously observed in three Aboriginal Australian individuals from Western Australia (Hudjashov, et al. 2007; Nagle, et al. 2017). We observed the M14 haplotype in a contemporary individual from the Western Central Desert region of Western Australia. Given these haplotypes were carried by just 2.1% of the total ancient and contemporary Aboriginal Australians included in this research these haplotypes appear very rare and limited to Western Australia.
Eleven M42* haplotypes were observed, representing 28.9% of the total haplotypes observed, and carried by 33 ancient and contemporary Aboriginal Australians (23.1%). The M42* broader haplogroup consists of two sub-groups: M42a* and the newly created M42c*. In this research, M42a* comprised five separate haplotypes (13.2% of the total haplotypes observed), and was carried by 18 ancient and contemporary Aboriginal Australians (12.6%). The geographic distribution of the M42a* haplotypes was restricted to the eastern states, with a very distinct northern Queensland and Riverine, New South Wales divide. Haplotype M42a1a was observed solely in Weipa, and M42a1b2 only in Cairns, present in both ancient and contemporary Aboriginal Australians. Haplotypes M41a1a1, M42a1b1, M42a1a2a and M42a3 were limited to the Riverine region of New South Wales.

The newly classified M42c* grouping comprised five separate haplotypes (13.2% of the total haplotypes observed), and was carried by 15 contemporary Aboriginal Australians (10.5%). Four of these haplotypes, M42c1, M42c1b, M42c2a1 and M42c2a2, were observed in single individuals in Weipa and Cairns in Queensland; Pilbara in Western Australia, and Barham in New South Wales. M42c1b1 was observed in 11 contemporary Aboriginal Australians from Esperance, the Western Central Desert and Pilbara in Western Australia. Nagle, et al. (2017) also reported a wide geographic distribution of both the M42a* and M42c* groups.

4.4.2 Lineages within macrohaplogroup N

Nine mitochondrial haplotypes (23.7% of the total haplotypes observed) belonged to macrohaplogroup N: two N13*, two O* and five S*. A total of 49 ancient and contemporary Aboriginal Australians carried these mitochondrial lineages (34.3%). The N13* mitochondrial haplotypes, including N13 and N13a, appear to be geographically restricted in terms of distribution and were only detected in four Aboriginal Australian individuals (2.8%). Mitochondrial haplotype N13 was only detected in contemporary individuals from Western Australia, specifically Kalumburu in the Kimberley and Wongatha. Haplotype N13a was present in both ancient and contemporary Aboriginal Australians from north-eastern Queensland in Cairns and
Mapoon. Similar results were reported in Nagle, et al. (2017), with N13, N13a and N13a1 present in individuals from Cairns and Western Australia.

O* mitochondrial haplotypes, including O1a1 and O2, accounted for 14.6% of the total mitochondrial genetic variation observed. O1a1 was found in 11 contemporary and ancient Aboriginal Australians from Western Australia (Wongatha, Ngadjumaya, Western Central Desert, Pilbara and Golden Ridge/Kalgoorlie), two individuals from Birdsville in western Queensland, and one from Yuendumu, Northern Territory. Haplotype O2 was only observed in Western Australia contemporary individuals. Tobler, et al. (2017) found no O* haplotypes in eastern Australia, while Nagle, et al. (2017) reported a wide distribution being present in Cairns and Ipswich in Queensland, Western Australia and the Northern Territory.

Five S* haplotypes were observed, representing 13.6% of the total haplotypes observed, and was carried by 24 ancient and contemporary Aboriginal Australians (16.8%). The S1a1 and S2b2 haplotypes we observed were shared between contemporary Aboriginal Australians from Western Australia and Birdsville, Queensland. This pattern was also observed with haplotype O1a1, with all contemporary Aboriginal Australians from Birdsville carrying what appear to be Western Australian specific haplotypes. However, Nagle, et al. (2017) report a wider distribution of both haplotypes with S1a1 and S2b2 being present at other Queensland locations.

In this research haplotype S2a1a was present only in the Riverine region of New South Wales and was not detected in any other geographic location. Though, Nagle, et al. (2017) reported its presence in two Queensland locations. S1a was carried by contemporary individuals from Yuendumu in the Northern Territory and the Riverine, New South Wales, and an ancient individual from Cairns, Queensland. While these three individuals represent just 2.1% of the total number of ancient and contemporary Aboriginal Australians included in this research, this haplotype appears to have a wide geographic range.

S5 was observed in just two contemporary Aboriginal Australian individuals from Pilbara and Kalumburu, Western Australia. These haplotypes were carried by just 2.1%
of the total ancient and contemporary Aboriginal Australians included in this research and therefore likely represent rare haplotypes limited to Western Australia.

4.4.3 Lineages within macrohaplogroup R

Fourteen mitochondrial haplotypes (36.9% of the total haplotypes observed) belong to macrohaplogroup R (a sub-group of macrohaplogroup N): eleven P* and three R*. A total of 55 ancient and contemporary Aboriginal Australians carried these mitochondrial lineages (38.5%).

Eleven P* haplotypes were observed, representing 28.9% of the total haplotypes observed, and was carried by 45 ancient and contemporary Aboriginal Australians (31.5%).

All but two P* haplotypes were found exclusively in the eastern states of Australia. The exceptions were haplotypes P3b2 and P8a that were recorded from Western Australia and the Northern Territory respectively. The geographic distribution of the P5* and P12* haplotypes, including P5a1, P5a2, P5b1 P12a1 and P12b, appears to be centred in northern Queensland, with all but one of the 26 ancient and contemporary Aboriginal Australians carrying these haplotypes originating from that region.

In this research haplotype P11a1 appears centred in Riverine, New South Wales. All but two of the 12 individuals come from this region, that of an ancient individual from Normanton, Queensland and a contemporary individual from Esperance, Western Australia. Nagle, et al. (2017) reported a wider distribution, with distribution in Queensland, Victoria and the Northern Territory.

Mitochondrial haplotypes within the broader R12* classification, including haplotypes R12, R12a1 and R12b, representing 6.9% of the total mitochondrial genetic variation and were carried by 10 individuals (7% of the total number of ancient and contemporary Aboriginal Australians included). All individuals carrying the R12* haplotypes were from Western Australia. Both Kivisild, et al. (2006) and Nagle, et al. (2017) also observed the presence of R12* haplotypes in Western Australia.
4.4.4 Overview of mitochondrial genomic variation

Analyses of the mitochondrial diversity of the 143 ancient and contemporary Aboriginal Australians included in this research shows both high levels of genetic diversity and geographic structure. The most common basal haplogroup was P* with 31% of the total haplotypes observed falling within that classification, followed by M* (incorporating M14, M15, both M42a* and M42c*) with 25% (Figure 37). Haplogroups N* and Q* (unlikely to be an Aboriginal Australian-specific haplogroup) were the least common with 2-3%.

Figure 37 | Overall observed mitochondrial haplogroup proportions

It is important to note that despite decades of Aboriginal Australian focussed mitochondrial research, only a small number of geographic locations have been covered to date. As these analyses have shown, the ancient and contemporary Aboriginal Australians we analysed resulted in both the discovery of a number of novel mitochondrial haplotypes, and the reclassification of many existing mitochondrial haplotypes. The absence of genomic data from much of the Australian continent significantly limits the inferences involving the presence, absence, frequency and distribution of Aboriginal Australian mitochondrial haplotypes. It can be expected that with each new study, the overall picture of the mitochondrial genetic diversity of Aboriginal Australians will continue to be more accurate.
4.5 Mitochondrial repatriation results

Ancient ANC

Ancient Aboriginal Australian ANC from Golden Ridge/Kalgoorlie, Western Australia belongs to the O1a1 mitochondrial haplotype (Figure 29). The larger phylogenetic clade representing the O1a1 haplotype consists of fourteen Aboriginal Australian individuals: ancient ANC; ten contemporary individuals from Western Australia (Pilbara, Wongatha, Golden Ridge/Kalgoorlie, Ngadjuamaya and the Western Central Desert); two from Birdsville, QLD, and one from Yuendumu in the Northern Territory (Figure 44). A smaller subclade that includes ancient ANC, also consists of contemporary individuals from Birdsville (Karnic), Wongatha (Wati), Western Central Desert (Wati), and Ngadjuamaya (Nyungar) (Figure 38 and Table 17).

![Figure 38](image)

**Figure 38** | Mitochondrial distribution of haplotype O1a1

Ancient individuals are represented by a red circle, contemporary individuals are blue. Numbers in parentheses indicate multiple individuals from that location carrying the same mitochondrial haplotype. The absence of a number indicates one individual.

As the provenance of ancient ANC is known, it might be argued that the remains could be repatriated to the Wati language group as the contemporary matches from that group represent 57.1% of the total matches in the smaller subclade, and match the major language subgroup assigned to ancient ANC (Table 18). However, the inclusion of the two contemporary Aboriginal Australians from Birdsville, Queensland and the Yuendumu individual complicates matters. Therefore, if these ancient remains were
unprovenanced, we could not say with any certainty who best to repatriate the remains to, only that it is possible they originate from Western Australia.

Ancient KAAKUTJA (BK1)
Ancient Aboriginal Australian Kaakutja from Bourke, New South Wales belongs to the M42a3 mitochondrial haplotype (Figure 29). This small phylogenetic clade consists of just two Aboriginal Australian individuals: ancient Kaakutja from Bourke, New South Wales, and a contemporary individual from the Riverine (DQ404442) (Figure 39, Tables 17 and 18).

![Figure 39 | Mitochondrial distribution of haplotype M42a3](image)

Ancient individuals are represented by a red circle, contemporary individuals are blue.

Both ancient Kaakutja and the contemporary individual belong to, or were assigned to, the same major language subgroup, Paakantji. Additionally, both individuals are separated by a geographic distance of less than 100 km. In this instance, the mitochondrial DNA of both ancient and contemporary Aboriginal Australians could assist with the repatriation of these ancient remains if they were unprovenanced, to within a 100 km region and a single language group.

Ancient Flinders Island (FLI2), Stanley Island (STI) and Normanton (NORA1)
Ancient Aboriginal Australians FLI2 from Flinders Island, STI from Stanley Island and NORA1 from Normanton, Queensland belong to the P5b1 mitochondrial haplotype (Figure 29 and Figure 40), and are the only Aboriginal Australians included in this research carrying that particular haplotype. If these ancient individuals were
unprovenanced, it would require a larger dataset of both ancient and contemporary Aboriginal Australian individuals in order to determine their likely origin using only mitochondrial data.

Figure 40 | Mitochondrial distribution of haplotype P5b1
Ancient individuals are represented by a red circle.

Ancient KP1, KP2 and WLH4

Ancient Aboriginal Australians KP1 and KP2 from Barham, and WLH4 from Willandra Lakes, New South Wales belong to the S2a1a mitochondrial haplotype (Figure 29). The larger phylogenetic clade consists of six Aboriginal Australian individuals: ancient KP1, KP2 and WLH4, and three contemporary individuals from the Riverine region (Willandra, Riverine and Barham) (Figure 41).
A smaller subclade containing ancient KP1, KP2 and WLH4 includes a contemporary individual from Barham (KPM04) (Figure 29). All Aboriginal Australians within this smaller clade belong to, or were assigned to, the Kulin language sub-group (Tables 17 and 18). Though these four individuals are separated by a geographic distance of approximately 234 kilometres. We could be confident that the mitochondrial DNA of both ancient and contemporary Aboriginal Australians could assist with repatriation if these remains were unprovenanced, to within a 234 km region and a single language group.

Ancient PA409.3

Ancient Aboriginal Australian PA409.3 from Cairns, Queensland belongs to the S1a clade (Figure 29). This small clade consists of just three Aboriginal Australian individuals: ancient PA409.3 (Paman) and two contemporary individuals from the Riverine, New South Wales (DQ404440 – Paakantji) and Yuendumu, Northern Territory (DQ404441 – non-Pama-Nyungan language group, Walpiri) (Figure 42, Tables 17 and 18).
Figure 42 | Mitochondrial distribution of haplotype S1a

Ancient individuals are represented by a red circle, contemporary individuals are blue.

However, given the wide distribution of haplotype S1a, if these ancient remains were unprovenanced, we could not say with any certainty to whom it would be best to repatriate the remains to using only mitochondrial data.

Ancient MH5 and MH11

Ancient Aboriginal Australians MH5 and MH11 from Mapoon, Queensland belong to the P3a clade (Figure 29 and Figure 43). These two ancient individuals represent the only Aboriginal Australians to carry this haplotype. Therefore, if these ancient remains were unprovenanced, it would require a much larger dataset of both ancient and contemporary Aboriginal Australian individuals in order to determine their origins.
Figure 43 | Mitochondrial distribution of haplotype P3a

Ancient individuals are represented by a red circle. Numbers in parentheses indicate multiple individuals from that location carrying the same mitochondrial haplotype.

Ancient MH1, MH6, MH8, PA409.1, WPAH4 and WPAH7

Ancient Aboriginal Australians MH1, MH6 and MH8 from Mapoon, PA409.1 from Cairns, and WPAH4 and WPAH7 from Weipa, Queensland belong to the P5a1a mitochondrial haplotype. In addition to these ancient individuals, this phylogenetic clade consists of a single contemporary individual from Weipa (WPA01) (Figure 29 and Figure 44). These Aboriginal Australians are separated by a geographic distance of approximately 636 kilometres, with all belonging to, or were assigned to, the Paman language sub-group (Tables 17 and 18).

If these remains represented unprovenanced remains, we could say based on our mitogenomic analyses that the remains are likely Cape York in origin, and belong to the Paman language group. However, based on geography alone, this would result in the problematic assignment of ancient PA409.1 to the Weipa region. Based on the information we were provided by the Queensland Museum (regarding the collection and origin of that ancient individual) this would be incorrect.
Ancient WPAH5
Ancient Aboriginal Australian WPAH5 from Weipa, Queensland belongs to the P7 mitochondrial haplotype (Figure 29 and Figure 45) and is the only individual to carry this haplotype. If this ancient individual was unprovenanced, it would require a larger dataset of both ancient and contemporary Aboriginal Australian individuals in order to determine their likely origin.
Ancient MH4, MH7 and MH9

Ancient Aboriginal Australians MH4, MH7 and MH9 from Mapoon, Queensland belong to the P12a1 mitochondrial haplotype. In addition to these ancient individuals, this phylogenetic clade consists of a single contemporary Aboriginal Australian contemporary from Weipa (WPA02) (Figure 29 and Figure 46).

![Figure 46 | Mitochondrial distribution of haplotype P12a1](image)

Ancient individuals are represented by a red circle, contemporary individuals are blue. Circles superimposed indicate both ancient & contemporary individuals in that location.

All Aboriginal Australians in this clade belong to, or were assigned to, the Paman language sub-group (Tables 17 and 18). Weipa and Mapoon are separated by a geographic distance of approximately 95 kilometres, so we could be confident that the mitochondrial DNA of both the ancient and contemporary Aboriginal Australians could assist with repatriation if these remains were unprovenanced to within a 95 km region and a single language group.

Ancient PA86

Ancient Aboriginal Australian PA86 from Cairns, Queensland belongs to the P12b mitochondrial haplotype. In addition to this ancient individual, this phylogenetic clade comprises six contemporary Aboriginal Australians also from Cairns (CAI01, CAI04, CAI05, CAI06, CAI08 and CAI09) (Figure 29 and Figure 47).
Figure 47 | Mitochondrial distribution of haplotype P12b

Ancient individuals are represented by a red circle, contemporary individuals are blue. Circles superimposed indicate both ancient & contemporary individuals in that location.

All Aboriginal Australians in this clade belong to, or were assigned to, the Paman language sub-group (Tables 17 and 18) and are from the same geographic location. We could be confident that the mitochondrial DNA of both ancient and contemporary Aboriginal Australians could assist with repatriation if these remains were unprovenanced to a precise geographic location and language group.

Ancient PA109

Ancient Aboriginal Australian PA109 from Cairns, Queensland belongs to the M42a1b2 mitochondrial haplotype. In addition to this ancient individual, this phylogenetic clade consists of a single contemporary individual also from Cairns (CAI03) (Figure 29 and Figure 48). All individuals in this clade belong to, or were assigned to, the Paman language sub-group (Tables 17 and 18) and are from the same geographic location. We could be confident that the mitochondrial DNA of both ancient and contemporary Aboriginal Australians could assist with repatriation if these remains were unprovenanced to a precise geographic location and language group.
Figure 48 | Mitochondrial distribution of haplotype M42a1b2
Ancient individuals are represented by a red circle, contemporary individuals are blue. Circles superimposed indicate both ancient & contemporary individuals in that location.

Ancient WPAH1, WPAH2, WPAH3 and WPAH6
Ancient Aboriginal Australians WPAH1, WPAH2, WPAH3 and WPAH6 from Weipa, Queensland belong to the M42a1a mitochondrial haplotype. In addition to these ancient individuals, this phylogenetic clade consists of a single contemporary individual also from Weipa (WPA03) (Figure 29 and Figure 49).

Figure 49 | Mitochondrial distribution of haplotype M42a1a
Ancient individuals are represented by a red circle, contemporary individuals are blue. Circles superimposed indicate both ancient & contemporary individuals in that location.
All individuals in this clade belong to, or were assigned to, the Paman language sub-group (Tables 17 and 18) and are from the same geographic location. We could be confident that the mitochondrial DNA of both ancient and contemporary Aboriginal Australians could assist with repatriation if these remains were unprovenanced, to a precise geographic location and language group.

Ancient MH2 and MH10
Ancient Aboriginal Australians MH2 and MH10 from Mapoon, Queensland belong to mitochondrial haplotype Q1+16223 (Figure 29 and Figure 50), and are the only two individuals to carry this haplotype. This haplotype carries motif mutations also present in haplotypes commonly found in New Guinea/Papua New Guinea, Melanesia and Vanuatu (Ingman and Gyllensten 2003; Pierson, et al. 2006; Duggan, et al. 2014; Nagle, et al. 2017).

![Figure 50 | Mitochondrial distribution of haplotype Q1+16223](image)

The ancient individual is represented by a red circle.

It is therefore unclear if Q1+16223 is a mitochondrial haplotype that is Aboriginal Australian specific, and may indicate Pacific ancestry. In order to repatriate these ancient individuals, it would require a larger dataset of both ancient and contemporary individuals from population groups such as Papua, Melanesia and Vanuatu.
Ancient MH3

Ancient Aboriginal Australian MH3 from Mapoon, Queensland belongs to the N13a mitochondrial haplotype. In addition to this ancient individual, this phylogenetic clade consists of a single contemporary individual from Cairns (CAI07) (Figure 29 and Figure 51). These individuals are separated by a geographic distance of approximately 683 kilometres. Both individuals within the N13a clade belong to, or were assigned to, the Paman language sub-group (Tables 17 and 18).

![Figure 51 | Mitochondrial distribution of haplotype N13a](image)

Ancient individuals are represented by a red circle, contemporary individuals are blue.

If these remains represented unprovenanced remains, we could say based on our mitogenomic analyses that the remains are likely Cape York in origin, and belong to the Paman language group. However, based on geography alone, this would result in the problematic repatriation of ancient MH3 to the Cairns region. Based on the information we were provided by the Queensland Museum (regarding the collection and origin of that ancient individual) this would be incorrect.

Ancient NORA4

Ancient Aboriginal Australian NORA4 from Normanton, Queensland belongs to the P11a1 mitochondrial haplotype (Figure 29). This large phylogenetic clade consists of twelve Aboriginal Australian individuals: ancient NORA4, ten contemporary individuals from New South Wales (Willandra, the Riverine and Barham – Kulin and
Paakantji language groups), and one from Esperance in Western Australia (Nyungar) (Figure 52). Due to the widespread distribution of the P11a1 mitochondrial haplotype if these ancient remains were unprovenanced, we could not say with any certainty who best to repatriate the remains to, only that it is possible they originate from the Riverine region of New South Wales.

Figure 52 | Mitochondrial distribution of haplotype P11a1
Ancient individuals are represented by a red circle, contemporary individuals are blue. Numbers in parentheses indicate multiple individuals from that location carrying the same mitochondrial haplotype. The absence of a number indicates one individual.
Table 20 | Summarised ancient Aboriginal Australian mitochondrial repatriation results

<table>
<thead>
<tr>
<th>Sample ID (Assigned Language Group)</th>
<th>Ancient Location</th>
<th>Haplotype</th>
<th>Contemporary Matches (Language Group)</th>
<th>Contemporary Location</th>
<th>Geography Based</th>
<th>Language Based</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancient ANC (Wati)</td>
<td>Golden Ridge/Kalgoorlie, WA</td>
<td>O1a1</td>
<td>BDV04 &amp; BDV08 (Karnic) WON02, WON3 &amp; WON05 (Wati) WCD06 (Wati) NGA02 (Nyungar)</td>
<td>Birdsville, QLD Wongatha, WA Western Central Desert, WA Esperance, WA</td>
<td>Crosses two states: WA &amp; QLD</td>
<td>Predominantly Wati, but also includes Karnic &amp; Nyungar</td>
<td>Does not assist with repatriation</td>
</tr>
<tr>
<td>Ancient Kaakutja (Paakantji)</td>
<td>Bourke, NSW</td>
<td>M42a3</td>
<td>DQ404442 (Paakantji)</td>
<td>Riverine, NSW</td>
<td>Within 100km</td>
<td>Paakantji</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Ancient FLI2, STI &amp; NORA1 (Paman)</td>
<td>Flinders Island, QLD, Stanley Island, QLD, Normanton, QLD</td>
<td>P5b1</td>
<td>No contemporary matches</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Requires a larger dataset</td>
</tr>
<tr>
<td>Ancient KP1, KP2 &amp; WLH4 (Kulin)</td>
<td>Barham, NSW</td>
<td>S2a1a</td>
<td>KPM04 (Kulin)</td>
<td>Barham, NSW</td>
<td>Approx. 234km</td>
<td>Kulin</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Ancient PA409.3 (Paman)</td>
<td>Cairns, QLD</td>
<td>S1a</td>
<td>DQ404440 (Paakantji) DQ404441 (Walpiri)</td>
<td>Riverine, NSW Yuendumu, NT</td>
<td>Crosses three states: QLD, NSW &amp; NT</td>
<td>Paakantji &amp; Walpiri</td>
<td>Does not assist with repatriation</td>
</tr>
<tr>
<td>Sample ID (Assigned Language Group)</td>
<td>Ancient Location</td>
<td>Haplotype</td>
<td>Contemporary Matches (Language Group)</td>
<td>Contemporary Location</td>
<td>Geography Based</td>
<td>Language Based</td>
<td>Conclusion</td>
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<tr>
<td>Ancient MH5 &amp; MH11 (Paman)</td>
<td>Mapoon, QLD</td>
<td>P3a</td>
<td>No contemporary matches</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Requires a larger dataset</td>
</tr>
<tr>
<td>Ancient MH1, MH6 &amp; MH8, PA409.1, WPAH4 &amp; WPAH7 (Paman)</td>
<td>Mapoon, QLD, Cairns, QLD, Weipa, QLD</td>
<td>P5a1a</td>
<td>WPA01 (Paman)</td>
<td>Weipa, QLD</td>
<td>MH1, MH6, MH8, WPAH4 &amp; WPAH7 – same location or 95km. PA409.1 approx. 636km</td>
<td>Paman</td>
<td>If repatriation was based on language, the correct group was identified. If based on geography, PA409.1 would be repatriated to the incorrect community</td>
</tr>
<tr>
<td>Ancient WPAH5 (Paman)</td>
<td>Weipa, QLD</td>
<td>P7</td>
<td>No contemporary matches</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Requires a larger dataset</td>
</tr>
<tr>
<td>Ancient MH4, MH7 &amp; MH9 (Paman)</td>
<td>Mapoon, QLD</td>
<td>P12a1</td>
<td>WPA02 (Paman)</td>
<td>Weipa, QLD</td>
<td>95km</td>
<td>Paman</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Ancient PA86 (Paman)</td>
<td>Cairns, QLD</td>
<td>P12b</td>
<td>CAI01, CAI04, CAI05, CAI06, CAI08 &amp; CAI09 (Paman)</td>
<td>Cairns, QLD</td>
<td>Same geographic location</td>
<td>Paman</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Sample ID (Assigned Language Group)</td>
<td>Ancient Location</td>
<td>Haplotype</td>
<td>Contemporary Matches (Language Group)</td>
<td>Contemporary Location</td>
<td>Geography Based</td>
<td>Language Based</td>
<td>Conclusion</td>
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<tr>
<td>Ancient PA109 (Paman)</td>
<td>Cairns, QLD</td>
<td>M42a1b2</td>
<td>CAI03 (Paman)</td>
<td>Cairns, QLD</td>
<td>Same geographic location</td>
<td>Paman</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Ancient WPAH1, WPAH2, WPAH3 &amp; WPAH6 (Paman)</td>
<td>Weipa, QLD</td>
<td>M42a1a</td>
<td>WPA03 (Paman)</td>
<td>Weipa, QLD</td>
<td>Same geographic location</td>
<td>Paman</td>
<td>Assists with repatriation</td>
</tr>
<tr>
<td>Ancient MH2 &amp; MH10 (Paman)</td>
<td>Mapoon, QLD</td>
<td>Q1+16223</td>
<td>No contemporary matches</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Requires a larger dataset of both ancient &amp; contemporary Aboriginal Australians, Papuans &amp; Melanesians</td>
</tr>
<tr>
<td>Ancient MH3 (Paman)</td>
<td>Mapoon, QLD</td>
<td>N13a</td>
<td>CAI07 (Paman)</td>
<td>Cairns, QLD</td>
<td>Approx. 683km</td>
<td>Paman</td>
<td>If repatriation was based on language, the correct group was identified. If based on geography, MH3 would be repatriated to the incorrect community</td>
</tr>
<tr>
<td>Sample ID (Assigned Language Group)</td>
<td>Ancient Location</td>
<td>Haplotype</td>
<td>Contemporary Matches (Language Group)</td>
<td>Contemporary Location</td>
<td>Geography Based</td>
<td>Language Based</td>
<td>Conclusion</td>
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</tr>
<tr>
<td>Ancient NORA4 (Paman)</td>
<td>Normanton, QLD</td>
<td>P11a1</td>
<td>KPM02 (Kulin), RIV02, RIV04, RIV05, RIV07 (Paakantji), RIV08 (Paakantji &amp; Karnic) &amp; DQ404444 (Paakantji) WIL01 (Paakantji), WIL02, WIL04 (Kulin) ENY08 (Nyungar)</td>
<td>Barham, NSW Riverine, NSW Willandra, NSW Esperance, WA</td>
<td>Crosses three states: QLD, NSW &amp; WA</td>
<td>Kulin, Paakantji, Karnic &amp; Nyungar</td>
<td>Does not assist with repatriation</td>
</tr>
</tbody>
</table>
4.4.1 Summary of mitochondrial repatriation results

The success of using mitochondrial DNA in the repatriation of unprovenanced ancient Aboriginal Australian remains has been mixed and is very much dependant on the distribution of the mitochondrial haplotype carried by ancient individuals (Table 20).

For two ancient Aboriginal Australians, ANC from Golden Ridge/Kalgoorlie, Western Australia and NORA4 from Normanton, Queensland, the mitochondrial haplotypes they carry (O1a1 and P11a1 respectively) are shared with contemporary individuals across three Australian states, and three or four different language subgroups. In both instances, the haplotype distribution is predominantly centred in a particular state or region (O1a1 in Western Australia, P11a1 in the Riverine, New South Wales) with just a few individuals sharing the same haplotype outside that region. As mentioned earlier in this study the area surrounding Birdsville was the main growing and processing centre for pituri, with Aboriginal Australians travelling long distances for their supplies (Aiston 1937; Barlow 1979; Kerwin 2010). It is possible that the distribution of these mitochondrial haplotypes is a reflection of pre-European trade and cultural trade, which brought together Aboriginal Australians from all over the continent. Alternatively, they could just occur in higher frequencies than other haplotypes. Whether these individuals have ancestral connections to these areas cannot be determined. In terms of repatriating these ancient individuals, it is not possible to determine their origin with mitochondrial DNA alone.

Four mitochondrial haplotypes, including eight ancient Aboriginal Australians (FLI2, MH2, MH5, MH10, MH11, NORA4, STI and WPAH5) require a larger dataset of both provenanced ancient and contemporary Aboriginal Australians in order to assess whether they can be repatriated using mitochondrial DNA. For haplotypes P5b1, P3a and P7, there were no contemporary Aboriginal Australian matches and appear to represent rare haplotypes. Ancient Aboriginal Australians MH2 and MH10 from Mapoon, Queensland may fall outside the scope of this research but within the mitochondrial distribution of Papua, Melanesia and Vanuatu. In order to repatriate these two ancient individuals, the comparative dataset would need to include mitochondrial sequences from these outside locations.
A large number of ancient Aboriginal Australian individuals were successfully matched to contemporary individuals from the same geographic location or region (within a 250 km range) or language sub-group. For ancient Aboriginal Australian individuals PA86 and PA109 from Cairns Queensland, the contemporary matches were also from the Cairns region, and the same language subgroup. This was also the case with ancient WPAH1, WPAH2, WPAH3 and WPAH6 from Weipa, Queensland who all matched with a contemporary Aboriginal Australian from the same geographic region.

For three mitochondrial haplotypes (M42a3, P12a1 and S2a1a) the geographic distribution appeared region-specific, with the ancient Aboriginal Australians matching contemporary individuals from the same language group present in the area in which the remains were recovered. For Kaakutja from Bourke, New South Wales (haplotype M32a3) the best genomic match was with a contemporary Paakantji individual from the Riverine region of New South Wales (DQ404442), within 100 km from Kaakutja’s burial location.

Similarly, the best genomic match for ancient Aboriginal Australians MH4, MH7 and MH9 from Mapoon, Queensland (haplotype P12a1) was with a contemporary Paman individual from Weipa, Queensland (WPA02), 95 km south of Mapoon. Lastly, KP1 and KP2 from Barham, New South Wales, and WLH4 from Willandra, New South Wales, formed a phylogenetic clade with a contemporary individual from Barham, New South Wales (KPM04), all within the Kulin language group.

However, any successes were undermined by two problematic mitochondrial matches. The first, haplotype N13a, consisted of just two individuals, ancient Aboriginal Australian MH3 from Mapoon, Queensland, and a contemporary individual from Cairns, Queensland (CAI07). Geographically, these two locations are some 683 km apart, though both fall within the same Paman language subgroup.

Secondly, haplotype P5a1a, consisted of a clade of six ancient Aboriginal Australians (MH1, MH6 and MH8 from Mapoon; WPAH4 and WPAH7 from Weipa; and PA409.1 from Cairns, Queensland) and a contemporary individual from Weipa (WPA01). Again, all of these individuals are from the same Paman language subgroup, however
geographically Mapoon and Weipa are 95 km apart, while Weipa and Cairns are 636 km apart. If geographic distance alone was used to provenance ancients MH3 and PA409.3, the remains would be returned to the incorrect Aboriginal Australian community. However, if language group was used, the correct language subgroup, Paman in both cases, would have been identified.

**Conclusion**

Despite the substantial losses and forced relocations endured by Aboriginal Australians over the last 200 years, analyses of the mitochondrial DNA of ancient and contemporary Aboriginal Australians reveals well defined structure, with many lineages or haplotypes being geographically restricted. A number of new mitochondrial subclades were discovered and amendments to existing Aboriginal Australian mitochondrial haplogroup classifications proposed as a result of analyses completed in this chapter.

The mitochondrial DNA of 31 ancient Aboriginal Australians was analysed, to assess whether this DNA could assist with the repatriation of unprovenanced ancient Aboriginal Australian remains. The results varied. For three ancient Aboriginal Australians (9.7%) mitochondrial DNA would not assist with repatriation as the haplotypes were common across a number of states or territories. A further eight ancient individuals (25.8%) had no contemporary Aboriginal Australian matches, and would require a larger comparative dataset of both Aboriginal Australians and their nearby neighbours from Papua New Guinea or the islands of the Torres Strait.

For the remaining 18 ancient individuals, whether mitochondrial DNA could assist with repatriation is dependent on the benchmark being used to assess the accuracy of the matches generated phylogenetically. If major language subgroup is used, then all 18 ancient Aboriginal Australians (58.1%) show that mitochondrial DNA can assist with repatriation, allowing their return to Country from the museums in which they are currently housed. However, if geography is used only 16 (51.6%) were accurate to within 100 km of the recorded burial location of the ancient Aboriginal Australians. Two ancient individuals, PA409.1 from Cairns and MH3 from Mapoon, Queensland, would be returned to the incorrect communities (6.5%).
As previously discussed, the return to Country for ancestral remains currently housed in museums nationally, as well as worldwide, is of paramount importance to many contemporary Aboriginal Australians and their communities. Repatriation to the incorrect Country would be extremely problematic. This, therefore, brings into question the reliability of using mitochondrial DNA for such an immense task, especially if a precise geographic location is required. However, if the result of using very strict benchmarks such as geographic location prevents ancestral remains from being returned at all, would Aboriginal Australian communities accept a less stringent benchmark such as major language subgroup? This decision is not for researchers to make.

It is important to remember that mitochondrial DNA represents a single locus inherited from just one parent, measuring just ~16.6 thousand base pairs within the ~3.2 billion base pair long nuclear genome. Whether the resolution of these mitochondrial results change with additional nuclear analyses will be determined in Chapter 5. However, due to the nature of ancient DNA preservation in ancient Aboriginal Australian skeletal remains, for many of the ancient remains included in this research mitochondrial DNA was all that can be recovered.
Chapter 5: Repatriation using nuclear DNA of Aboriginal Australians

This chapter is presented in the form of a manuscript and accompanying supplementary intended for submission for publication as a Research Article in Science Advances. The manuscript focusses on answering the question of whether it is possible to use mitochondrial and nuclear DNA of ancient and contemporary Aboriginal Australians to facilitate repatriation, a ‘Return to Country’, of the many sets of ancestral remains housed in museums worldwide.

This manuscript, inevitably, is repetitious in some respects, covering much of the material already discussed in Chapters 1 to 4, however presents new nuclear DNA analyses. I have included a cut down supplementary that only includes information not already covered in earlier Chapters.

I will be the first author of this output, and my role in its preparation is outlined below. Science recommends authors follow the defined CASRAI Contributor Roles Taxonomy (CASRAI 2018) when communicating author contributions in generating research outputs.

- Conceptualisation – I contributed to the formulation of the overarching research goals and aims of this work.
- Methodology – I helped develop and design the methodology followed in this research in respects to sequencing strategies, sampling plans and the types of analyses completed.
- Validation – I helped verify and validate the results obtained. This was completed through manual checks of sequencing data, contamination estimation and ancient DNA authentication processes.
- Formal Analysis – I processed initial sequencing data, generated summary statistics, and performed mitochondrial and nuclear analyses.
- Investigation – I conducted experiments in the laboratory which resulted in the successful recovery of mitochondrial and nuclear genomes included in this research.
- Resources (Samples) – I liaised with Aboriginal Australian communities and collected saliva samples that were included in the contemporary DNA dataset.
• Writing (Original Draft) – Much of the original drafts originated from writing contained within this thesis. I was assisted with additional writing of these drafts by my supervisor Professor David Lambert, Dr Sally Wasef and Associate Professor Martin Sikora.

• Writing (Review & Editing) – I led the preparation of the manuscript for submission to Science, and assisted with incorporating the critical input provided by all other authors.

• Visualisation – I prepared phylogenetic trees incorporated in this manuscript, and assisted with generating the map within.

• Data Curation – I am jointly responsible for managing and maintaining the research data with Professor David Lambert.

5.1 Manuscript: Ancient nuclear genomes enable repatriation of Indigenous human remains

Authors: Joanne L. Wright¹, Sally Wasef², Tim H. Heupink¹,³, Michael C. Westaway¹, Simon Rasmussen³, Colin Pardoe⁴, Gudju Gudju Fourmile⁵, Michael Young⁶, Trish Johnson⁷, Joan Slade⁸, Roy Kennedy⁹, Patsy Winch¹⁰, Mary Pappin snr¹¹, Tapij Wales¹², William ‘Badger’ Bates¹³, Sharnie Hamilton¹⁴, Neville Whyman¹⁵, Sheila van Holst Pellekaan¹⁶,¹⁷, Peter J. McAllister¹⁸, Paul S.C. Taçon¹, Darren Curnoe¹⁹, Ruiqiang Li²⁰, Craig Millar²¹†, Sankar Subramanian¹,²²†, Eske Willerslev²₃,²₄,²⁵†, Anna-Sapfo Malaspinas²₃,²₆†, Martin Sikora²₃*, David M. Lambert¹*  

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⁶ Barkandji/Paakantyi Elder, Red Cliffs, VIC, Australia.
⁷ Barkandji/Paakantyi Elder, Pooncarie, NSW, Australia.

8 Ngiyampaa Elder, Ivanhoe, NSW, Australia.
9 Ngiyampaa Elder, Hay, NSW, Australia.
10 Mutthi Mutthi Elder, Balranald, NSW, Australia.
11 Mutthi Mutthi Elder, Mildura, VIC, Australia.
12 Thanynakwith Elder, Napranum, QLD, Australia. (deceased December 2017).
13 Barkandji/Paakantyi Elder, Mildura, VIC, Australia.
14 Barapa Barapa Elder, Barham, NSW, Australia.
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5.1.1 Abstract: After European colonisation, the ancestral remains of Indigenous people were often collected for scientific research or display in museum collections. For many
decades Indigenous people, including Native Americans and Aboriginal Australians, have fought for their return. However, many of these remains have no recorded provenance, making their repatriation very difficult or impossible. In order to determine whether DNA-based methods could resolve this important problem, we sequenced ten nuclear genomes and 27 mitogenomes from ancient pre-European Aboriginal Australians (up to 1,540 yr BP) of known provenance and compared them to 100 high coverage contemporary Aboriginal Australian genomes, also of known provenance. We report substantial ancient population structure showing strong genetic affinities between ancient and contemporary Aboriginal Australian individuals from the same geographic location. Our findings demonstrate the feasibility of successfully identifying the origins of unprovenanced ancestral remains using genomic methods.

Introduction: Over many decades, there has been a concerted effort by Aboriginal Australians to reach agreement with museums that would enable their ancestral remains to be returned to the original communities from which they were taken (Langford 1983; Webb 1987; Pardoe 1991a; Truscott 2006; Pardoe 2012a). This issue is of particular importance to Aboriginal Australians given their spiritual connection to the place in which they were born and lived (Grieves 2009). Many Aboriginal Australians believe that in order for their ancestor’s spirits to rest, their remains must be returned to their ancestral lands and their kin after death (Truscott 2006). For Aboriginal Australians this is termed return to ‘Place and Country’.

Unfortunately many of these remains have no specific details regarding their geographic origin, tribal affiliation or language group (Hanchant 2004; Pardoe 2013). For many, the only information provided is that such remains are ‘Aboriginal Australian’ in origin. This
lack of detailed information regarding their provenance means that many remains are unable to be returned. Museums and other institutions are unable to repatriate remains without first identifying the appropriate communities or custodians (Simpson 2001). Significantly, this problem is not limited to Aboriginal Australians, but is a worldwide issue affecting almost all Indigenous groups.

Recent advances in ancient DNA methods and bioinformatics suggest that a genomic approach can be successfully used to facilitate, on a large scale, the identification of unprovenanced remains. The success of such an approach has been illustrated by the identification and subsequent repatriation of the 8,500 year old Native American remains of “Kennewick Man” (Rasmussen, Sikora, et al. 2015). To date, there have been a limited number of genomic studies of ancient Aboriginal Australians with only two focused on pre-European (Adcock, et al. 2001; Heupink, et al. 2016a), and two on post-European remains (Rasmussen, et al. 2011; Tobler, et al. 2017).

The first ancient DNA study of Aboriginal Australians, published in 2001, reported the recovery of ten short mitochondrial sequences (Adcock, et al. 2001). However, a later genomic study of the same ancient remains established that the earlier sequences were likely PCR artifacts (Heupink, et al. 2016a). The latter study reported the recovery of the complete mitogenome of an ancient Aboriginal Australian (labelled WLH4) (Heupink, et al. 2016a). This was the first example of the recovery of authentic human DNA from an Australian archaeological context, proving it was possible to successfully recover ancient DNA, despite the harsh Australian climatic conditions and resulting poor DNA preservation. However, the recovery of the ancient nuclear genomes of Australia’s First People has been to date elusive.
Results

Samples, sequencing and authenticity

In collaboration with Aboriginal Australian Traditional Owners and communities across Australia, we undertook ancient DNA analyses of 27 sets of remains from archaeological excavations of known Aboriginal Australian burial sites or previously repatriated remains of known provenance.

The distinction between Aboriginal Australian genomes from pre- and post-European contact periods is important for the determination of the provenance of remains. This is because the resulting admixture can confound the determination of provenance (Malaspinas, et al. 2016). The ages of the skeletal remains of the ancient Aboriginal Australians studied here were determined from either archaeological or $^{14}$C dates (Supplementary Materials). Different regions of Australia were settled by Europeans at different times. However, all of the ages of ancient samples pre-date European contact thus excluding the possibility of admixture. This distinction is further supported by the presence of morphological and pathological characteristics including the absence of dental caries and distinctive tooth wear patterns typical of hunter-gatherer diets (Littleton 2018). The hair samples used were from individuals born prior to European settlement of the geographic regions sampled (Extended Data Table 1 and Supplementary Materials).

Using DNA in-solution capture methods and second-generation sequencing we successfully recovered ten ancient nuclear genomes (0.3x - 6.9x coverage) and 27 mitogenomes (2.3x - 321x coverage) from pre-European contact remains of Aboriginal Australians (dated 95 – 1,540 yr BP). Additionally, for four of the male ancient Aboriginal Australians (KP1, MH8, PA86 and WLH4) we were able to recover partial or complete Y-chromosome sequences (haplogroups S1a and S1c). Preliminary shotgun
sequencing showed that many of the ancient samples had very low levels of endogenous DNA (Extended Data Table 2). We therefore designed and used a modified method that employed whole genome capture baits (MYbaits) to enrich targeted nuclear sequences. We found that by modifying the hybridisation temperature to 57°C, the genome coverage obtained was significantly enhanced.

All recovered ancient sequences exhibited damage patterns characteristic of ancient DNA, with elevated levels of cytosine to thymine misincorporations in the 5’ end of fragments, and guanine to adenine misincorporations in the 3’ end (Methods). Additionally, contamination estimates for both mitochondrial and genome-wide sequences all displayed low contamination levels (Methods).

**Establishing comparative contemporary DNA datasets**

We used the recovered ancient Aboriginal Australian mitogenomes and nuclear genomes as proxies for unprovenanced remains to determine whether we could accurately identify their geographic origins using DNA-based methods.

While we successfully recovered four partial or complete Y-chromosomes from ancient male Aboriginal Australians, previous research of contemporary Aboriginal Australian males (Taylor, et al. 2012; Nagle, et al. 2015; Malaspinas, et al. 2016) have shown considerable levels of Eurasian admixture, with large numbers of research participants carrying non-Indigenous Y-chromosome haplotypes. The level of Eurasian admixture observed in contemporary Aboriginal Australian males varies greatly, with between ~32% and ~70% being observed in different regions of Australia (Taylor, et al. 2012; Nagle, et al. 2015; Malaspinas, et al. 2016). Undeniably, there has been a significant loss of Aboriginal Australian Y-chromosome genetic diversity since European settlement,
perhaps with entire lineages lost to the past. This Y-chromosome admixture makes it extremely difficult to obtain a clear picture of the paternal genomic history of Aboriginal Australians and as such, would hinder attempts to find possible ancestral connections for repatriation purposes.

We constructed comparative contemporary mitochondrial and nuclear DNA datasets based on self-reported language group affiliations (Bowern and Atkinson 2012; Malaspinas, et al. 2016), as well as geographic locations (Fig. 1). The contemporary nuclear DNA dataset comprised 100 high coverage nuclear genomes of Pama-Nyungan language speaking Aboriginal Australians. A total of 112 mitogenomes showing Aboriginal Australian specific mitochondrial haplogroups were included in the mitochondrial DNA analyses, including 17 previously published genomes (van Holst Pellekaan, et al. 2006; Hudjashov, et al. 2007; Rasmussen, et al. 2011) (Extended Data Table 3).

As these contemporary datasets were assembled for the purpose of repatriation, they required a high degree of accuracy. Therefore, only previously published contemporary DNA sequences of known geographic origin and/or language group were used (van Holst Pellekaan, et al. 2006; Hudjashov, et al. 2007; Rasmussen, et al. 2011; Malaspinas, et al. 2016). The recent publication of 111 mitogenomes recovered from historic hair samples from locations in Queensland and South Australia were not included, as the deposited sequences lacked precise geographic identifiers (Tobler, et al. 2017).

**Mitochondrial genetic affinities**

Using mitochondrial Maximum Likelihood phylogenetics (Fig. S2), we compared 29 ancient Aboriginal Australian mitogenomes (Rasmussen, et al. 2011; Heupink, et al.
2016b) with the 112 contemporary mitogenomes we previously assembled (Fig. 2). We observed 38 distinct mitochondrial haplogroups, with novel subclades discovered within mitochondrial haplotypes M42c*, R12a*, R12b* and M42a3, while new subtypes were found for most other mitochondrial haplotypes (Supplementary Materials). For 18 ancient Aboriginal Australian individuals (62.1%), the closest contemporary match was an individual from the same geographic region (within 235 km). Within this group, nine ancient individuals could be matched to a contemporary individual within 100 km, and six matched to individuals from the same location from which the ancient remains originated (Fig. 1).

For the remaining 11 ancient individuals (37.9%) the results were either inconclusive due to a lack of contemporary matches or because some mitochondrial haplotypes were geographically widespread. It has been previously documented that some Aboriginal Australian mitochondrial haplotypes have widespread distributions across the continent, while others are regional-specific (van Holst Pellekaan 2013a; Malaspinas, et al. 2016; Nagle, et al. 2016; Nagle, et al. 2017; Tobler, et al. 2017), reflecting ancient female migration patterns. While this is an interesting anthropological finding, it is less helpful for our specific purpose. In two instances (6.9%), the closest ancient mitochondrial matches were not from the same geographic locations. In this case, the closest contemporary matches were contemporary individuals from opposite from sides of Cape York Peninsula, some 635 km away (Fig. 1). As the return to ‘Place and Country’ of ancestral remains is of paramount importance to many contemporary Aboriginal Australian communities, repatriation to an incorrect Country would be problematic. Therefore, the use of mitochondrial DNA alone is not recommended for repatriation.
Nuclear genetic structure of ancient and contemporary populations

Subsequently, we performed a series of analyses on the ten ancient Aboriginal Australian nuclear genomes recovered. To investigate the overall genetic structure of ancient and contemporary Aboriginal Australian populations, we analysed the individuals in the context of a reference panel including 2,117 modern individuals from worldwide populations genotyped for 593,610 SNPs. Principal component analysis and supervised model-based clustering (ADMIXTURE) revealed high levels of recent admixture across many Aboriginal groups, in particular those from Bourke (BKM) and Willandra Lakes (WIL) (Fig. 3). While most of the recent admixture is European in origin, we also observed evidence of East Asian gene flow, particularly among individuals from north Queensland (CAI, WPA). In contrast, individuals from the Western Central Desert (WCD) were almost completely unadmixed and were therefore subsequently used as a reference group for Aboriginal Australian ancestry in local admixture inference and masking, using previously described methods (Malaspinas, et al. 2016). All of the ancient Aboriginal Australian samples were found to cluster close to the unadmixed WCD individuals (without apparent European admixture), as expected.

Nuclear genetic affinities

We investigated the genetic relationships among the ancient Aboriginal Australian individuals using both PCA and outgroup $f_{st}$-statistics. These analyses revealed substantial genetic structure between individuals from different geographic regions, with three distinct clades observed (Fig. 4A and B). To further characterise their relationships, we fitted the highest coverage ancient individual from each region onto an admixture graph using $q$Graph (Fig. 5). We found that the deepest divergence separated the ancient individual from Kalgoorlie/Golden Ridge (ANC) from all remaining individuals.
Importantly within the eastern clade, we identified a trifurcation among the three major geographic regions (Fig. 5) without any apparent closer relationship between the groups from north-western (MH8, WPAH4) and north-eastern Queensland (PA86) with respect to the individuals from New South Wales (WLH4, KP1) (Fig. 5). Notably, we detected ~13% Papuan-related ancestry in the individual from Cairns (PA86). This was also observed for contemporary individuals from the same region (20).

We next sought to determine whether the ancient Aboriginal Australian individuals were most closely related to individuals with known traditional connection to the same region, thereby facilitating repatriation. Genetic clustering using PCA or outgroup $f_1$-statistics both suggested a higher genetic affinity of the ancient individuals to local contemporary groups, compared to contemporary Aboriginal Australians from other geographic locations (Fig. 4). We further investigated these patterns using $f_4$-statistics in the form of $f_4$(Mbuti,Ancient;Contemporary,Papuan) on the masked dataset. This measured the amount of excess allele sharing of an ancient Aboriginal Australian individual with a given contemporary group, when compared to Papuans. We found that the local contemporary groups consistently showed the highest level of sharing with the respective ancient Aboriginal Australian individual, supporting long term local population continuity (Fig. 6). For the two higher coverage ancient Aboriginal Australian individuals (KP1, MH8) we additionally carried out haplotype-sharing analyses. As previously supported with the allele-frequency based results, the largest excess haplotype sharing for both KP1 and MH8 was also with the local contemporary group (Extended Data Fig. S1).
Discussion

Our analyses of the first ancient nuclear genomes of Aboriginal Australians reveals substantial past population structure. This result confirms the previous identification of an east versus west genetic divide between the contemporary Australia populations (Malaspinas, et al. 2016) while, at the same time, revealing further major geographic subdivision. When combined with the strong genomic affinities observed among ancient and contemporary populations from the same geographic locations, we showed that we could use these findings to reliably repatriate ancient unprovenanced remains to the correct Place and Country.

Over a long period, mitochondrial and Y chromosome sequences have proved to be highly informative genetic markers for a diverse array of applications. This includes phylogenetic reconstruction, the timing of divergent events and tracing the spread of humans worldwide (Nielsen, et al. 2017). However, some researchers, as a result of a biogeographic study of mitochondrial DNA diversity (Tobler, et al. 2017), have proposed that such genetic marker sequences can be used to facilitate repatriation. However, in contrast, one of the major findings of the current study is that mitochondrial DNA sequence variation performs poorly in this regard. Our results suggest that mitochondrial sequences, if used in repatriation efforts in the Australian context would result in a significant percentage (~7%) of remains being returned to the wrong Indigenous group, with consequent disastrous effects.

We show that even in the arid conditions of Australia, low coverage nuclear genomes can be recovered and, more importantly, these low coverage genomes can be used to precisely and accurately repatriate ancient remains. Furthermore, with advances in DNA capture and recovery methods, as well as with improvements Single Nucleotide Polymorphism
analyses and the decreasing costs of genome sequencing, this general approach is likely to become more affordable and effective over time. We propose that our approach can be used now and will be used routinely in the future to return remains to their rightful kin. Finally, our findings suggest that a similar approach could be used to facilitate the repatriation of Indigenous remains in other countries. This would represent a major scientific and social advance and would go some way to ameliorating the negative effects of past European colonization.

**Materials and Methods**

**Ancient DNA laboratory methods**

All pre-PCR procedures were carried out in a dedicated Ancient DNA facility in the Australian Research Centre for Human Evolution, Griffith University. The facility is sealed, geographically isolated from any modern molecular laboratory, has one-way airflow under positive pressure, and the air is HEPA filtered. The skeletal remains and hair samples were processed within a UV sterilised ULPA-filtered vertical laminar flow cabinet (used for this purpose only). Each sample was initially treated with 10% bleach to remove any surface contaminants and then washed with UltraPure DNase/RNase-Free Distilled Water (Invitrogen) to remove any remaining bleach.

Skeletal material was processed using a Dremel rotary tool with a high-speed diamond cutter head or manually with a sterilised scalpel blade, where the outer surface was discarded. DNA was extracted from ~50mg of bone or tooth powder as previously described (Heupink, et al. 2016a). Extraction blanks were included throughout all procedures. Hair samples were processed in 2-4 mL of digestion buffer as previously described (Rasmussen, et al. 2011). This solution was incubated in a rotating incubator oven for 24 hours at 45 °C. After complete digestion, the samples were centrifuged at
9000g for 3 min. The supernatant was combined with 10x volume of a modified binding buffer (500 mL of Qiagen PB buffer, 1:250 pH indicator I, 15 mL 3M pH5.2 of NaOAc and 1.25 mL NaCl). Extractions were purified using the MinElute Reaction Cleanup Kit (Qiagen) following the manufacturer’s protocol and eluted using 100 μL EB buffer (Qiagen) after incubation for 10 min at 37 °C.

**Ancient DNA library construction, amplification and screening**

Double-stranded Illumina DNA libraries were built according to the methods previously described (Meyer and Kircher 2010; Rasmussen, et al. 2011; Heupink, et al. 2016a), with some minor modifications in the *Taq* polymerase used for amplification. Libraries built from different samples were amplified using different polymerases (Extended Data Table 2). All libraries were screened on a Bioanalyzer 2100 (Agilent) to ensure that the DNA length distributions did not show any significant artifacts from amplification e.g. artificially long molecules due to serial binding or primer dimers. Where these problems occurred, the number of PCR amplification cycles or primer concentration was adjusted. All PCR and extraction blanks were screened for contaminant library constructs on the Bioanalyzer.

**Whole-genome in-solution target capture**

Between 100 and 500 ng of library amplified DNA was generated as described above using multiple secondary amplifications, some of which were sent for direct sequencing. DNA libraries were subjected to custom MYbaits whole human genome capture (ArborBio). Target capture enrichment was performed according to manufacturer’s instructions however hybridisation was performed for 36 to 42hrs at 55°C to 57°C. The bead binding buffers, initial 30 min incubation and cleaning steps were also performed at
this chosen hybridisation temperature. Post-capture libraries were amplified on binding beads using Kapa HiFi Uracil + (Kapa Biosystems) according to the MYbaits manual (version 3) for between 14 and 17 cycles.

**Ancient sequencing**

Ancient samples were sequenced using 100 base pair single end reads. This sequencing was conducted using either a HiSeq 2500 Sequencing System (Illumina) at The Danish National High-Throughput DNA Sequencing Centre in Copenhagen or on a MiSeq Sequencing System (Illumina) using 150 v3 kits at the Griffith University DNA Sequencing Facility. Sequences were base-called using CASAVA 1.8.2 (Illumina).

**Contemporary sequencing**

DNA library construction and sequencing of contemporary samples was conducted at the Kinghorn Centre for Clinical Genomics at the Garvan Institute in Sydney, Australia or Novogene Bioinformatics Technology Corporation Limited in Beijing, China. Sequencing libraries were generated using Truseq Nano DNA HT Sample Preparation Kit (Illumina, USA) following the manufacturer’s recommendations. Libraries were then 150 base pair paired-end sequenced on an Illumina HiSeq X. Genome coverage of this sequencing averaged between 45-60x.

**Ancient DNA mapping and consensus calling**

Adapters were trimmed from the sequencing data using fastx_clipper, part of FASTX-Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/) using parameters -Q 33 –l 30. Levels of human DNA were determined by mapping reads to the human reference genome (GRCh37/hg19) or the revised Cambridge reference mitochondrial genome (Andrews, et al. 1999). Mapping was completed using BWA 0.6.2 (Li and Durbin 2010)
with the following options: seed disabled (Schubert 2012) with terminal low quality trimming (-q 15), before being aligned using BWA-0.6.2 aln with seed disabled. The mapped reads were sorted, duplicates removed and merged using SAMtools 0.1.18 (Li, et al. 2009; Li 2011a).

**Contemporary data processing**

The paired-end contemporary DNA sequences were mapped to the human reference genome (GRCh37/hg19) or the revised Cambridge reference mitochondrial genome (Andrews, et al. 1999) using BWA 0.6.2 (Li and Durbin 2010). Duplicate sequences were then removed from Alignment/Map (SAM) files using SAMtools (Li, et al. 2009; Li 2011a). Using the mpileup command, with a maximum depth parameter of 1000, variant call format (VCF) files were generated for each chromosome separately. Using an awk command indel variations were excluded. The VCF files of individual modern genomes were merged using VCFtools (Danecek, et al. 2011) after zipping and indexing using tabix.

**Mitochondrial Maximum Likelihood phylogenetics**

Consensus mitogenomes were generated and ambiguous bases were checked and manually corrected. Mitochondrial haplotypes were identified using HaploGrep 2.0 software (Weissensteiner, et al. 2016), with mitochondrial variations described in PhyloTree mtDNA build 17 (van Oven and Kayser 2009). Alternatively haplotypes were identified manually and novel ones were named in accordance with recent Aboriginal Australian mitochondrial haplogroup classifications (Nagle, et al. 2017).

All mitogenomes were aligned using SeaView 4.6.1 (Gouy, et al. 2010). The mitochondrial evolutionary history of Aboriginal Australians was inferred using the
Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) with 1000 bootstrap replications, as implemented in MEGA7 (Kumar, et al. 2016). The tree with the highest log likelihood (-19648.2315) was used (Fig. 2). Initial tree(s) for the heuristic search were obtained by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (categories +G, parameter =). This rate variation model allowed for some sites to be evolutionarily invariable ([+I], % sites).

The final tree was drawn to scale, with branch lengths measured in the number of substitutions per site and involved 141 mitochondrial sequences. All positions containing gaps and missing data were eliminated, and a total of 11,042 nucleotide positions were used in the final dataset. Subsequent annotation and presentation of the tree were completed using Interactive Tree of Life (iTOL) 3.4.3 software (Letunic and Bork 2016).

**Analysis panel**

Genotyping of newly sequenced as well as previously reported modern individuals was carried out using samtools/bcftools (Li, et al. 2009) followed by filtering as previously described (Sikora, et al. 2017). For population genetic analyses, those genotypes were merged with a reference panel of 2,286 modern individuals from world-wide populations, genotyped at 593,610 SNPs using the Affymetrix Human Origins array (Lazaridis, et al. 2014; Lazaridis, et al. 2016). Ancient individuals were represented by pseudo-haploid genotypes obtained by sampling a random allele at each SNP position. We additionally carried out diploid genotyping as described above for two ancient samples with higher coverage: KP1 (6.9x) and MH8 (6.8x).
Population structure and admixture modelling

Principal component analysis (PCA) was carried out using smartpca (Patterson, et al. 2006), by projecting ancient individuals onto the components inferred from modern individuals using the ‘lsqproject’ option. Genetic affinities of ancient and modern individuals were investigated using the $f$-statistic framework (Patterson, et al. 2012). We used ‘outgroup $f_3$’ statistics to determine the amount of shared genetic drift between pairs of individuals and/or groups, as well as $f_4$ statistics for allele sharing symmetry tests (Extended Data Tables 5 and 6). Model-based clustering implemented in ADMIXTURE was used to investigate patterns of recent admixture, in supervised mode using European (French), East Asian (Han), Oceanian (Papuan) and Australian (WCD) individuals as putative source populations.

Local ancestry inference

Local ancestry deconvolution of the modern individuals was carried out using RFmix (Maples, et al. 2013) and a panel of four reference populations: European (French), East Asian (Han), Oceanian (Papuan) and Australian (WCD). Prior to this analysis, we subsampled each reference population to the number of individuals observed in the smallest population (WCD; 12 individuals) in order to avoid potential bias due to unbalanced panel sizes. A “masked” dataset was then obtained by restricting the analysis to SNPs for individuals that were homozygous for Australian ancestry (WCD).

Haplotype sharing analyses

Haplotype sharing among modern Australians and the two highest coverage ancient individuals (KP1, MH8) was inferred using CHROMOPAINTER (Lawson, et al. 2012). Haplotype phase was reconstructed for the full set of individuals with diploid genotypes.
using Shape-IT (Delaneau, et al. 2013). We then performed chromosome painting for the two ancient individuals as recipients, using all modern Australians, as well as selected outgroups (French, Han, Papuan, Bougainville) as potential donors. Differential sharing for the pair of ancient individuals was quantified using the symmetry statistic (Skoglund, et al. 2015):

$$ S (A, B) = \frac{Donor\ A - Donor\ B}{Donor\ A + Donor\ B} $$

Standard errors were obtained using a block jacknife across the 22 autosomes.

**Ancient DNA authentication**

Recovered ancient DNA sequences were authenticated using a number of methods. Firstly, DNA damage patterns were estimated for each sample using MapDamage software (Jónsson, et al. 2013). Samples showed a mean fragment length of 49.2-97.4 base pairs, with higher fragment lengths observed in the better-preserved hair samples. All samples exhibited damage patterns characteristic of ancient DNA, with elevated levels of cytosine to thymine misincorporations in the 5’ end of fragments, and guanine to adenine misincorporations in the 3’ end (Dabney, et al. 2013) (Extended Data Table 2).

**Ancient DNA contamination estimates**

Mitochondrial contamination estimates were obtained using the contDeam command in the schmutzi software package (Renaud, et al. 2015) and endogenous consensus sequences were generated using default settings. Both the schmutzi generated consensus sequences and the original ancient sequences were then manually checked using the
SAMtools tview command (Li, et al. 2009). For genome-wide sequences, DNA sequencing libraries were screened using contamix (at the time of mapping against the reference genome). All libraries with >3% contamination were excluded from further analyses.

Supervised admixture of the ancient nuclear genomes was undertaken using five putative ancestry sources (Extended Data Figure S2). Low levels of contamination from a European source were observed in some of the low-coverage ancient samples such as PA109. Though it has been reported that using ADMIXTURE on low-coverage samples can result in statistical uncertainty associated with SNP and genotype calling, resulting in high error rates due to sampling, alignment and sequencing errors (Nielsen, et al. 2012). Our additional analyses using PCA confirmed all ancient individuals cluster tightly with previously described Aboriginal Australians without recent admixture (WCD). Therefore, this contamination can be attributed to a combination of the <3% previously observed, as well as low-coverage of the nuclear genome.

**Ancient DNA sex determination**

Sex determination was carried out using the method previously described (Skoglund, et al. 2013), comparing the morphological and archaeological information provided with each set of remains. In all instances, the assigned sex was as expected, which also rules out contemporary contamination from members of the opposite sex.

**Acknowledgments:** We thank all of the Aboriginal Australian participants and our Traditional Owner collaborators for supporting this research. Thank you to Shaun Adams for allowing us access to remains he excavated and associated community reports. We thank the National Throughput DNA Sequencing Centre at the University of
Copenhagen, Novogene Bioinformatics and Kinghorn Centre for Clinical Genomics for DNA sequencing; Gareth Price (Queensland Facility for Advanced Bioinformatics) for bioinformatics assistance; Alison Devault ( ArborBio) for target capture advice; Nano Nagle and John Mitchell (La Trobe University) and Mannis van Oven (Erasmus MC University) for assistance with the identification of novel mitochondrial haplotypes. Thank you to Ron Galway and Norman Moore for assistance with this work in Barham, NSW. This research was conducted using the NeCTAR Research Cloud supported by the Queensland Cyber Infrastructure Foundation. Funding: This work was supported by the Australian Research Council (DP140101405, DP110102635, LP120200144, LP140100387 and LP130100748). J.L.W. was supported by the Australian Government, the Environmental Futures Research Institute and Australian Research Centre for Human Evolution with a PhD scholarship.

at the European Genome-phenome Archive (EGA, http://www.ebi.ac.uk/ega/), which is hosted by the EBI, under the accession number XXXXXXXXXX. Ancient Aboriginal genome sequence data have been deposited in the Genbank database (accession numbers XXXXXXXX to XXXXXXXX).
Fig. 1. Details of the locations and language groups of Aboriginal Australian samples.

Yellow shading indicates the distribution and location of Pama-Nyungan language families. Orange shading indicates the distribution of non-Pama-Nyungan language families. Dashed lines show the approximate distribution of accepted major language subgroups as published in (20) with language names in italics. Red symbols indicate previously published mitochondrial or nuclear genomes; blue indicates new unpublished data. Circles indicate contemporary Aboriginal Australian samples and stars represent ancient individuals. Sample code abbreviations have been included in parentheses.
Fig. 2. Mitochondrial Maximum Likelihood phylogeny of ancient and contemporary Aboriginal Australian mitogenomes.

Mitochondrial Maximum Likelihood phylogenetic relationships among ancient subgroups (bold) and contemporary Aboriginal Australians. Coloured segments indicate separate mitochondrial haplogroups.
Fig. 3. Genetic structure of ancient and contemporary Aboriginal Australians.

A) Shows the first two principal components of a PCA of individuals from non-African populations, with ancient individuals (black outlines) projected. B) Supervised admixture of contemporary Australians using five putative ancestry sources. Many modern Australians show evidence for European (French; orange stars) or East Asian (Han; blue diamonds) admixture. All ancient individuals cluster tightly with previously described Australian Aboriginals without recent admixture (Western Central Desert; WCD).
Fig. 4. Genetic affinities between ancient and contemporary Aboriginal Australians.

A) Modern Australians projected onto a PCA inferred from the five higher coverage ancient individuals covering all geographic regions sampled. Inset shows full PCA including ancient individuals, larger plot shows zoomed region of modern individuals only (dashed box in inset). Polygons and large symbols indicate the range and median of the principal components for each modern population, respectively. B) Multidimensional
scaling (MDS) based on pairwise genetic drift sharing (outgroup $f_{st}$ statistics) between ancient individuals and modern populations (masked dataset). The results highlight the considerable genetic structure among ancient Aboriginal Australians. In both analyses, modern individuals show closest affinities with ancient individuals from the same geographic region.

Fig. 5. The best-fitting admixture graph model of relationships among ancient Aboriginal Australia genomic sequences.

Shown is an admixture graph fit ($\max |Z| = 2.88$) including the highest coverage ancient individual from each region and group. Individual PA86 from Cairns in north-eastern Queensland could only be fit as a mixture with $\sim13\%$ Papuan-related ancestry.
Fig. 6. Allele sharing between ancient and contemporary Aboriginal Australians.

Each panel shows $f_4$-statistics of the form $f_4\text{(Mbuti,Ancient;Modern,Papuan)}$. Negative values indicate the amount of excess allele sharing of the respective ancient individual with a contemporary Australian group (y axis) compared to Papuans (masked dataset). Error bars show three standard errors obtained from a block jacknife. Contemporary groups are sorted according to the amount of excess allele sharing in each panel. Notably, ancient individuals show the highest amount of sharing with their respective local contemporary group.
Extended Data Figure S1: Local continuity between ancient and contemporary groups. Chromosome painting symmetry statistics, testing for excess of haplotype donations of contemporary groups to one of the two ancient individuals. Results show that ancient individuals receive increased haplotype donation from their local modern groups but are symmetric with respect to more distant populations.

Extended Data Figure S2: Supervised admixture of ancient Aboriginal Australians using five putative ancestry sources. Many ancient Australians show evidence for Papuan (light blue) admixture. Ancient individuals showing low level contamination from European sources (French; orange) have low nuclear coverage.
Chapter 6: Summary

6.1 Is this a new model for repatriation-based research in Australia?

The past injustices and trauma suffered by Aboriginal Australians due to the actions and policies implemented in Australia have left gaps in the self-knowledge of many contemporary Aboriginal Australians. This loss of cultural heritage and history led to many of the Traditional Owners who were involved in this research seeking to learn more about their history through the analyses of ancestral remains as well as contemporary genomes of First Australians generally. Due to the significance of the repatriation issue to many Aboriginal Australians, it was unsurprising then that they also wished to directly test whether DNA, both their own and that of their ancestors, might help facilitate a return to Country of unprovenanced remains from museums worldwide.

To test whether DNA could facilitate the repatriation of ancestral remains, reference datasets of mitochondrial and nuclear contemporary Aboriginal Australian genomes were constructed and compared with DNA recovered from the ancient Aboriginal Australian individuals. These analyses showed substantial population structure. While a distinct east versus west Australian population divide was previously observed in contemporary individuals (Malaspinas, et al. 2016), both mitochondrial and nuclear analyses reported here showed that this population structure exists even at a regional level. This indicates long term genomic continuity within the regions of Australia and as such supports the view that Aboriginal Australians have occupied the same regions for centuries.

Mitochondrial phylogenetic analyses reveal the majority of mitochondrial haplotypes observed are region-specific allowing connections between ancient and contemporary Aboriginal Australians to be made. A small number of haplotypes discovered were either geographically widespread, appearing across a number of Australian states, or rare haplotypes for which there were no contemporary matches. For these ancient remains it is impossible to determine their origin using mitochondrial DNA alone. It seems inevitable that the current mitochondrial haplogroup classification system will continue to evolve with future research and further resolution may be gained.
It was possible to successfully determine the origin of 58.1% of the ancient Aboriginal Australians included in this research using mitochondrial DNA alone. However, this success was weakened by the problematic results obtained for two ancient individuals PA409.1 (Cairns) and MH3 (Mapoon), with their respective contemporary matches living on the opposite side of Cape York Peninsula. As discussed previously in this thesis, the return of ancestral remains to the incorrect Country would be a concern of Aboriginal Australian communities. Therefore, given these two false positive results, only detected because their provenance had already been established, the reliability of using mitochondrial DNA alone for repatriation is significantly undermined and must be questioned. If this method is to be utilised in the future, it is recommended that it be used with great caution and if possible in conjunction with other tools or methods to help verify the results.

By far the most accurate results obtained were using nuclear DNA, working in 100% of cases and to precise geographic locations. This result was supported by a number of different statistical analyses: PCA, f3- and f4 outgroup statistics, and chromosome painting symmetry statistics. Each of these analyses independently showed population structure and local continuity between the ancient and contemporary populations in each geographic location. When combined, these nuclear analyses provide strong evidence that nuclear DNA, as a tool for repatriation, is very effective and if applied to unprovenanced ancestral remains could assist with their repatriation.

6.2 Potential obstacles

It is important to note that there are three major obstacles that need to be overcome before this proposed repatriation method can be put into practice. The first is the cost involved in completing this type of work, which comprises two major components. Firstly, the work must be undertaken in specially designed clean laboratories in order to mitigate the threat of contamination. This includes the necessity for trained ancient DNA researchers employed to undertake the work, both in terms of laboratory work and bioinformatics analyses. Ancient DNA research can be extremely difficult even for those trained in the field.
While the cost of sequencing contemporary genomes continues to decrease over time, the cost of both extracting and sequencing ancient DNA remains expensive, especially if nuclear DNA is the target of interest. Many museums worldwide may argue, understandably, that they are already struggling for funding in a world where visits to their institutions by the public are declining, and funding for this sort of research is well outside their reach. Therefore, funding would need to be sourced from relevant government organisations.

Currently, in order for repatriation to occur museums need to establish appropriate custodians. Therefore, this DNA–based repatriation model poses a catch 22 in that the work likely cannot be undertaken without permission from the appropriate Aboriginal Australian communities, and until the provenance of remains is determined these communities cannot be identified. This means that despite this tool being effective, it might not ever be used, leaving ancestral remains in limbo until a way forward can be negotiated.

Finally, the last obstacle is the potential response of the Aboriginal Australians impacted by the use of this proposed DNA-based repatriation tool. While it is clear that the repatriation of ancestral remains from museums is a significant issue for Aboriginal Australians and something they have been fighting for decades, the work is by nature both destructive and invasive. The Aboriginal Australian communities involved in this research understood this and accepted that it was a necessary evil in order to test a model designed to solve an important social problem. However, other Aboriginal Australians may be less accepting of the destruction of small amounts of their ancestors remains, even if it results in their return to Country. However, throughout this research the vast majority of Traditional Owners were typically very positive about this issue.

6.3 Recommendations and future work

6.3.1 National discussion

Given the complexity of the obstacles that must be addressed before this DNA-based repatriation tool might be applied, the way forward is through discussions involving all stakeholders. These discussions should occur at a national level, between the Australian Government, specially appointed Aboriginal Australian representatives, and museums.
Discussions at this level will allow all parties to decide how best to move forward and how the tools presented in this research should be utilised. Ultimately, this decision is for Aboriginal Australians to make. Obviously, any such research will need to find a balance between Aboriginal Australian’s cultural concerns and the potential benefits that will come from repatriation of ancestral remains. It is of paramount importance that before work commences discussions involve expectations in terms of results. As the work involves ancient DNA, it is entirely possible that some, even many, ancestral remains will not yield useful DNA or may be contaminated, resulting in no answer.

### 6.3.2 Regional or national repatriations

As shown by the mitochondrial component of this research, there are instances in which ancient individuals carried haplotypes which were regional-specific, but these regions have sometimes encompassed hundreds of kilometres, and comprised many distinct, smaller language and cultural groups. For such ancestral remains, or for those for which there are not sufficient genomic data to allow assignment of an origin (but are nevertheless known to be Aboriginal Australian) a possible solution would be the establishment of appropriate Keeping Places.

The concept of Keeping Places is not new, with discussions on the topic dating back many years (Simpson 2006; Tuniz, et al. 2016). In fact, several Indigenous communities across Australia have already established Keeping Places within their Country. For example, the Kimberley Aboriginal Law and Cultural Centre (KALACC) in north Western Australia have constructed Keeping Places in three separate locations, with plans to construct a further two in the near future. The KALACC also plans to develop a ‘Regional Resting Place’ in which they can appropriately store remains that have poor provenance (Kimberley Aboriginal Law and Cultural Centre n.d). These ideas could be extended to state and/or national level, with Keeping Places established in each state or a centralised national location that is culturally significant to all Aboriginal Australians. These locations would act as storage facilities for ancestral remains whose provenance could not be sufficiently determined using current technology or existing datasets.

The establishment of such facilities would go a long way to solving a major component of Aboriginal Australian discontent over the continued housing of remains in museums.
A return, albeit even only back to Australia generally, their spiritual homeland, is a far more palatable solution for Aboriginal Australians than allowing them to remain in museums. One consistent message we received from Traditional Owners, specifically from collaborative partners, throughout the five years this research took to complete, was that Aboriginal People wish to be in control of their own culture, heritage and future. They want the ability to make decisions regarding the handling of their ancestral remains and more broadly about their heritage. It would therefore be important that these facilities be operated by the local Aboriginal communities themselves. Young Aboriginal Australians would then be given employment opportunities and to learn about their culture and heritage, archaeology, science and stories, especially given how much has been lost since European settlement some 230 years ago.

6.3.3 Increased sample sizes

This research has resulted in substantial results using the datasets able to be constructed. However, an obvious extension of the research would be to further increase the size of the datasets with the inclusion of additional provenanced ancient and new contemporary genomes from geographic locations not yet sampled. For mitochondrial analyses, this is particularly important. Given the number of mitochondrial haplotypes observed, it is apparent that we have not yet reached peak understanding of the mitochondrial diversity of Aboriginal Australians, and a larger sample size could help resolve some of the unanswered questions faced. Also, given the Oceanic admixture we observed particularly in Aboriginal Australians from North Queensland, it is recommended that the dataset also include genomes, both nuclear and mitochondrial, from individuals from neighbouring regions such as Papua and the Torres Strait.

6.3.4 Stolen Generation

Another potential use of the datasets assembled could be to provide valuable insight into the kinship between contemporary Aboriginal Australians, particularly those of the Stolen Generation. Many Aboriginal Australians were removed forcibly from their families and/or Countries, and have consequently lost their pasts. It is possible that the use of the contemporary genomic dataset could facilitate the identification and the reconnection of kin. However, it is one thing to analyse the DNA of people from the past, but for living
people the impact of the results obtained and the connections identified may result in additional hurt and harm. The analyses will reveal information about more than just the single consenting participant. It could change the stories people have been told over the years, and potentially sever the connections that people have with each other and their Country. There has been an interest in the use of these data to address this social issue including special interests among Aboriginal Australian communities and Australian Government departments specialising in reconnecting members of the Stolen Generation with their lost families. The question is, should this tool be used and by whom? Finally, this question can only be answered by the Aboriginal Australian People themselves.

6.3.5 Repatriation toolkit

Despite the successful results obtained in this research, it is recommended that DNA as a tool for repatriation be incorporated into a 'repatriation toolkit'. DNA should be used in conjunction with existing repatriation methods, namely extensive archival research, craniometrics and stable isotope analyses. DNA studies of Aboriginal Australians is still in its infancy, and as we have seen, genomic technology has advanced from PCR amplification of mitochondrial control regions to whole genome sequencing in just a decade. Technology is advancing at such a rate that in a few years it may be possible to recover shorter fragments of ancient DNA, allowing recovery from samples that failed in this previous research.

One method which may prove useful is using single-stranded DNA library preparation for samples that failed to yield DNA when the double-stranded method was used. This method has been successfully used for the recovery of ancient genomes of archaic humans such as the Denisovans (Meyer, et al. 2012) and Neandertals (Prüfer, et al. 2014), which are typically highly fragmented. Standard ancient DNA library preparation usually involves ligation to double-stranded DNA fragments, however with the single-stranded method each of the two DNA strands are incorporated into the DNA library as separate strands (Gansauge, et al. 2017). These strands are normally lost during subsequent purification steps, and for some samples the single-stranded method has shown an increase in the proportion of endogenous DNA recovered when compared with double-stranded libraries with some samples (Bennett, et al. 2014; Gansauge, et al. 2017). However, single-stranded DNA library preparation is costly in terms of both labour and expense (Gansauge, et al. 2017).
6.4 Significance of this research

In addition to developing an effective tool for provenancing ancestral remains for repatriation, this research is significant in a number of other ways, the first is potentially the most important. This research was driven by Aboriginal Australian Traditional Owners and their communities. Their desire to learn more about themselves developed into five years of research in which they were actively involved and equal partners in the direction the work took. Without their input and knowledge this research would not have been possible. This is a significant shift from Aboriginal Australians being scientific subjects, as they were in the past, to them being researchers in their own right.

A result of assembling the datasets and testing the repatriation tool is that our understanding of Aboriginal Australian genomic diversity expanded considerably. To date there have been only a few DNA-based studies of Aboriginal Australians and through this process previously unobserved mitochondrial haplotypes have been discovered. This has resulted in novel haplotypes being identified and a reclassification of the existing Aboriginal Australian mitochondrial haplotypes. It is clear there is still much to learn in this regard, with mitochondrial genetic diversity being very high.

Previous ancient DNA studies of Aboriginal Australians comprised a single nuclear genome of a 100-year old hair sample from Golden Ridge/Kalgoorlie, Western Australia (Rasmussen, et al. 2011); the mitogenome of a pre-Holocene dated ancient man, WLH4, from Willandra, New South Wales (Heupink, et al. 2016b); and a number of historical hair samples from South Australia and Queensland collected between 1928 and 1939 (Tobler, et al. 2017). Therefore, the research presented here is significant as it presents the 10 ancient nuclear genomes (up to 1600yrBP) and 30 additional ancient Aboriginal Australian mitogenomes. This contributes substantially to the existing genomic data available for both ancient and contemporary Aboriginal Australians. These genomic sequences have the potential to contribute significantly to future research involving the archaeology, anthropology and linguistics of Aboriginal Australians, and more broadly the migration of modern humans into this part of the world.
6.5 Final remarks

This research provides a possible way forward in the repatriation debate that has caused many Aboriginal Australians considerable frustration for decades. If agreement can be reached amongst the stakeholders regarding how it should be implemented, it has the potential to assist in bringing closure to a painful chapter of history for many Aboriginal Australians. While it cannot, and will not correct the mistakes of the past, it may provide healing through the eventual return to Country for many lost ancestors.
Appendices/Supplementary items

Appendix 1: Heupink et al. 2016

Appendix 2: New DNA study confirms ancient Aborigines were the First Australians

**THE CONVERSATION**

**New DNA study confirms ancient Aborigines were the First Australians**

June 8, 2016 4.57pm AEST

The original excavation of Mungo Man, found near Lake Mungo in southwestern New South Wales, Australia, Wilfred Shawcross., Author provided

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**The Conversation's partners**

View partners of The Conversation

The question of whether Aboriginal People were the First Australians may be unanimously accepted today but research published back in 2001 suggested the contrary.

A new study out this week shows how we re-examined the research and our results put an end to that controversy.

The 2001 study, by the Australian National University's Gregory Adcock and colleagues, attained international significance, although its conclusions have remained controversial.

The authors published what was argued to be DNA sequences from Mungo Man, the oldest Australian, and from the remains of nine other ancient Aboriginal Australians.

One of the most important landmark claims by the authors was that the ancient DNA
sequence of Mungo Man showed high divergence from the sequences of contemporary humans, including those from Aboriginal Australians.

Hence they suggested that Mungo Man belonged to an early human lineage, which is not related to modern Aboriginal Australians.

An ‘older’ First Australian?

The implications of these claims were profound. They suggested multiple waves of migration to Australia, with an older more divergent population, which included Mungo Man, being replaced by a more recent population consisting of contemporary Aboriginal Australians.

The paper supported the hypothesis of modern human origins of the late Alan Thorne, who was also from ANU and an author of the paper.

Thorne had argued that Aboriginal origins were the result of two independent migrations, one of which was from a population whose origins were firmly linked with ancient Java (Indonesia).

The report of an extinct modern human lineage recovered from ancient DNA, he argued, supported his views on modern human origins. He said that we could no longer consider modern human origins to be solely African.

Naturally, the suggestion that contemporary Aboriginal Australians were not the true First Peoples of Australia resulted in heated debate.

The scientists who had done the research had not necessarily said exactly this. But this was how it was being portrayed, including on the front page of The Australian newspaper.

These controversial findings were further challenged by other researchers who questioned the authenticity of the sequences reported. They highlighted problems with the methods used to analyse the DNA sequences they recovered and there were concerns about the validity of the conclusions by the authors.

It is worth noting that Adcock and his colleagues rebutted some of these criticisms. They defended their conclusions about modern human origins, particularly with regard to the phylogenetic placement of Mungo Man.

Re-testing the results

Given the obvious importance of these claims, particularly in terms of the social question of who the First Australians really were, it deserved serious re-evaluation. This was possible using more recent advances in DNA methods and powerful analytical approaches.
To assess the results obtained by the original research we were given consent from the Willandra Lakes World Heritage Area Aboriginal Elders Committee – comprising the Parkindji, Ngiyampaa and Muthi Muthi elders – to re-sample material from this important fossil series.

This research, published this week in the Proceedings of the National Academy of Sciences, disputes these earlier claims.

Using more advanced second-generation DNA sequencing methods provides strong evidence that the DNA sequences originally reported by Adcock and colleagues were likely artefacts of the method used.

The polymerase chain reaction (PCR) technique used in the original study can produce unique hybrid molecules from different DNA templates. Whatever, the sequences reported previously were certainly not from Aboriginal Australians.

This new study was unable to replicate a single sequence published in the earlier study, with the exception of a sequence listed as belonging to Adcock himself, despite using some of the original extracts.

Finding contamination

The sample from Mungo Man that was re-sequenced in the new study contained sequences from five different European people, clearly representing contamination that may be due to handling throughout the years.

Among the other remains analysed was one man who was buried close to the location where Mungo Man was originally interred. The present study successfully recovered ancient DNA from this man. It represents the first recovery of a mitochondrial genome from an ancient Aboriginal person who lived before the arrival of Europeans.

The mitochondrial sequence was found to belong to a haplotype (grouping of human populations based on mitochondrial DNA) S2. This is exclusively found among contemporary Aboriginal Australian populations.

The present study is important for many reasons, but perhaps most importantly it has been planned and conducted, and is published, with the support of the Barkindji, Ngiyampaa and Muthi Muthi indigenous groups.

Finally, this new study refutes the earlier suggestion that another extinct lineage of people predated Aboriginal Australians. The archaeology and the genetics provide very strong evidence that our First People have been here over the past 50,000 years. This was long before people first arrived in Europe.
Appendix 3: Heupink et al. associated press


Appendix 4: Malaspina et al. 2016

Appendix 5: DNA reveals a new history of the First Australians

To coincide with the publication of “A Genomic History of Aboriginal Australia” we wrote a plain English companion article for The Conversation. Published on 22 September 2016, to date (as at 15 January 2017) the article has been read, blogged and shared to various social media platforms by 100,549 readers from across the world.

Understanding the history of Aboriginal Australians, their origins and how their population changed over some 50,000-plus years has always been an enormous challenge.

Many Aboriginal people have their own origin stories. Gujidgi, a Ginyu Yidinji Elder from the rainforest people around Cairns, says:

*The story which has been passed down from generations tells of three migrations that have occurred over many thousands of years, one of us coming to this ancient land first, then another as a period after the last Ice Age which saw the formation of the Great Barrier Reef, the other is of a migration out of Cairns that went back through the Cape into the Torres Strait to PNG and further."

Equally, scientific narratives of Aboriginal origins have presented different accounts. But these have been difficult to establish in part due to the difficulty and limitations of the science involved.

It’s also because of the social context that both science and archaeology work in within Australia.

The first few decades of modern archaeological research into Australia’s ancient past was conducted with very little to no involvement of Aboriginal Australian people.

This was followed by decades of debate over ownership of the past. Initial DNA research proposals floundered because little to no consultation was undertaken.

A new period of community based research with Aboriginal people was forged through the *sensitive and highly consultative approach* pioneered by geneticist Sheila Van Holst Pellekaan. Her work with Aboriginal people set the standard for later scientific studies in Australia.

We can now provide an example of work undertaken in partnership with Aboriginal Australian people from all parts of Australia, from the deserts to urban and regional centres. The details of the research are published today in *Nature*. 
The First Australians

Our results show very clearly that Aboriginal Australian people living today are the descendants of the First People to enter Australia, who lived between 25,000 to 40,000 years ago.

There is substantial evidence of admixture or intermingling with Asian, Oceanic and European people within the last 200 years. But in the Aboriginal DNA is an ancient story of migration into this continent, far deeper in time than any other population group has so far revealed.

It shows ancient contact and gene flow between the ancestors of the First Australians and now extinct populations of Neanderthals and Denisovans. This is very similar to the gene flows reported between Neanderthals, Europeans and Asians.

Our paper supports the results of earlier genomic research. This includes the foundational study on the first Aboriginal Australian genome from Western Australia and ancient DNA recovered from fossil remains from Lake Mungo.

Our research discounts the political agenda of some individuals who have claimed that Aboriginal Australians may not have been the First Australians.

The research also helps clarify a number of key points that archaeologists have been debating since the 1960s, such as where the ancestors of Aboriginal Australian people likely first entered the continent.

The first migration

There has been considerable debate as to whether the First Australians took the northern route (through Papua and then down into Cape York) or a more southerly route, crossing from Timor into north-western Australia.

Our evidence reveals a picture of population expansion from north-east Australia.

Around 10,000 years ago, sea levels rose and the land bridge between Papua and Cape York was flooded. Based on this, the genetic separation of Papuans and Aboriginal Australians was generally believed to have been initiated after this time.

But using large-scale genome data from Australians and Papuans we estimate the time of divergence between the two groups to be 37,000 years ago. This is much earlier than previously predicted.

This also suggests that barriers to intermarriage between Australia and Papua occurred much earlier than the creation of the barrier of Torres Strait.

Interestingly a significant barrier to gene flow within Australia also seems to have occurred at the time of the last great ice age, known as the Last Glacial Maximum (LGM).

East and west Australians
We see significant divergence between Aboriginal Australian people of north east and south west Australia. These groups are more genetically different than, for example, Native Americans and Siberians are from each other.

They are all Aboriginal Australians of course, but the onset of the LGM seems to have limited gene flow between east and west. As a result, the formation of a different population structure began some 81,000 years ago.

Further subdivisions among eastern and western Aboriginal Australians appeared to have occurred later. The phylogenetic relationships based on our genetic data correlated well with the divisions based on the Aboriginal (Pama-Nyungan) languages spoken by the people.

Some researchers have maintained for many years that the archaeological record shows significant population expansion in the last few thousand years before the arrival of Europeans.

Other archaeologists have disagreed, stating that demographic expansion can be a very difficult thing to prove from a record of carbon dates, stone tools and shell middens.

These signatures are very prone to destruction by such things as erosion and sea level change. Much of the first coastline initially colonised by the First Australians now lies beneath the waves, locked in a drowned landscape.

The genetic evidence for population increase in north east Australia, one area that some archaeologists have argued was subject to significant population expansion, is actually earlier than expected. It seems to begin some 10,000 years ago, which is several thousand years earlier than evidence provided by archaeology.

Also our genome data do not show any significant gene flow events into Australia from India around 6,000 years ago, suggested by previous research.

Desert life

What we did find was unique genetic variations specific to Aboriginal Australians that might have given them an improved ability to withstand cold and dehydration – potential adaptations to life in the desert.

Previous studies have shown the potential of DNA in understanding the ancestral relationships of Aboriginal Australian people. Genome sequencing in the past few years has revealed a far more complicated picture than first thought.

We can now tackle questions that have been debated for decades using the actual evidence from the biology of the First Australians.

It is crucial that we continue to make sure that such research is done in partnership with Aboriginal Australian people.

As noted by one of the key researchers in the project, Dr Craig Muller, who said:

"We have developed close relationships with many individuals throughout the project, and collaborated closely with Aboriginal Elders in each language group who provided important cultural information. The Elders also guided us to the appropriate people to participate."

A great example of how things have changed since the early years is that there are now new generations of young Aboriginal Australian researchers undertaking DNA research.

In parallel is the interest shown by many indigenous groups who are interested in this research and keen to partner with us. This suggests a wonderful future might lie ahead.
Aboriginal leader Cudjugudju co-authored this article.
Appendix 6: Malaspinas et al. associated press and recognition

In December each year, the editors and writers of the journal Science review the scientific accomplishments of the previous twelve months and select a Breakthrough of the Year winner, along with nine runners-up. Our research, published in Nature, was chosen as one of the ten scientific Breakthroughs of the Year for 2016.


Copyrighted material: Science 23 December 2016. The runners-up.
Appendix 6: Informed consent package

The peopling of Australia
INFORMATION SHEET

Who is conducting the research?

Principal investigator:
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Representatives for sample collection:
Dr Michael Westaway
Griffith University

Dr Craig Moller
University of Copenhagen

Dr Colin Pardoe
Australian National University

Ms Joanne Wright
Griffith University

Ms Jennifer-Leigh Campbell
Griffith University

Additional researchers are involved in other aspects of the research.

Why is the research being conducted?

This study investigates the history of Aboriginal and Torres Strait Island People in Australia. This is done by characterising the DNA of both contemporary people and those that lived up to 45,000 years ago. We will compare the DNA of all these individuals and also compare it with other people from all over the world. We aim to investigate the origin of the First Peoples of Australia and study any subsequent migrations within, to and from Australia. We will also investigate how ancient and contemporary Australians compare and are related to each other. These genetic data will also reveal how and when other populations have been in contact with Australian Aboriginals. The study of both modern and ancient Australian Aboriginals may also reveal how the Australian Peoples interacted with each other and how cultures and technologies were exchanged within Australia. This research will not investigate disease related questions.

What you will be asked to do

DNA is a molecule that contains the genetic information, describing much of an organism or individual. DNA exists throughout the body, particularly in cells, some of which get deposited in the saliva. The bulk of a person’s DNA has been inherited from both parents, the DNA therefore not just reveals information about the individual but also about the parents, grand parents and earlier ancestors.

DNA will be collected using spit sample kits, this is an hygienic and safe way to collect DNA. A funnel helps you deposit your saliva in a collection tube. After having deposited sufficient saliva (up to the fill line) the funnel can be discarded and the tube capped and deposited in the collection box. The sealed tube will be transported to the Brisbane laboratory where it will be prepared for shipment to our colleagues in Copenhagen and Beijing. There will be no other transfer of samples.

The DNA is multiplied through amplification to create enough synthetic DNA for future analyses and is stored in a freezer. The original and the copies of your DNA will be stored for a maximum of 10 years and are destroyed afterwards. The genome, a person’s complete set of genetic material, will be characterised using technology available in Copenhagen and Beijing. After characterising the genome it will be analysed by members of the research team. The genome will be compared with that of other people from other groups and areas and with ancient First Australians. The relation of this DNA will reveal how people and populations are related and may reveal when and where they migrated to and from.
The basis by which participants will be selected or screened

We are particularly interested in obtaining DNA samples from those individuals whose immediate ancestors (parents and grand parents) are most likely of direct Australian Aboriginal descent. These results provide us with the most information about the history of the Australian Peoples. It is for this reason we will ask about your direct ancestry. It is voluntary to provide this information. We are not able to accept samples from minors.

The expected benefits of the research

This study may reveal the history of Australia’s First People in that it may indicate their origins and how they interacted with other people in other parts of the world. We aim to investigate the number of individuals and populations that gave rise to Australia’s First People. We will also study the ancient migrations of these individuals and populations and their ancestors within and outside of Australia. In addition the study may reveal how certain cultural traditions and technologies have been exchanged across Australia. The project also holds the potential to create a DNA map of Australia’s First People and help identify the origin of Aboriginal skeletal remains that are being returned to Australia by museums.

Risks to you

The saliva sampling kit we use prevents any potential risk to you. With regards to privacy please refer to the following section.

Your confidentiality

DNA can also hold information that can be considered more private, for example in relation to genetic disease (although we will not investigate this aspect). The sample you give is immediately de-identified, the sample tubes are mixed with others and your consent form is kept separately in a locked box. A third party will ensure the consent forms are kept safe and separate from the samples afterwards. This de-identification ensures to the maximum extent that all results are published and reported anonymously and can not be re-traced to the individual. Despite our careful de-identification, your characterised genetic material is in principle re-identifiable. This means that someone with access to the data could in theory link the DNA data to you, despite the de-identification. We try to prevent this from happening in every possible way. The resulting de-identified data are available for researchers wishing to verify the results of this study only with an ethics approval. Any other research will have to be approved first by you, then by the research team and an appropriate ethics committee.

Your participation is voluntary

You are advised that your participation is voluntary and are free to decline without giving reasons. Also, if you agree to participate, you are free to withdraw from the study at any time, at which point your DNA will be destroyed. An independent third party will hold files that enable the cross referencing of names to individual samples, so that these can be destroyed in the event that a participant wants to withdraw from the study. Please contact Dr Donald R. Love at the Auckland City Hospital, New Zealand on donaldj@adcch.govt.nz or +64 9 307 4949 22013 in the event that you would like to withdraw from this study. Dr. Donald R. Love is not part of the research group, but an independent third party that will look after the consent forms and the numbers that associate these with the samples in the laboratory.

Questions / further information

You can contact any member of the research team that is present when you receive this sheet with questions. You can also contact the Chief Investigator at any other point in time, contact information is provided above.

The ethical conduct of this research

Griffith University conducts research in accordance with the National Statement on Ethical Conduct in

Human Research. If you have any concerns or complaints about the ethical conduct of the research project you should contact the Manager, Research Ethics on (07) 3735 4375 or research-ethics@griffith.edu.au.

Feedback to you

The results obtained from this study will be published in peer-review scientific journals. As a result of the de-identification we cannot report any individual or family results back to you or report any medical results to you. Instead, we will provide the representatives of your community with a plain language report that is available to those interested.

Privacy Statement

The conduct of this research involves the collection, access and/or use of your identified personal information. The information collected is confidential and will not be disclosed to third parties without your consent, except to meet government, legal or other regulatory authority requirements. A de-identified copy of this data may be used for other research purposes. However, your anonymity will at all times be safeguarded. For further information consult the University’s Privacy Plan at http://www.griffith.edu.au/about-griffith/privacy/publications/griffith-university-privacy-plan or telephone (07) 3735 4375.
The peopling of Australia - CONSENT FORM

Research team principal investigators:
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Prof Eske Willerslev Centre for GeoGenetics University of Copenhagen, Denmark +45 35338444 ewillerslev@snm.ku.dk
Dr Michael Westaway Environmental Futures Research Institute Griffith University, Australia +61 (0)7 373 59218 m.westaway@griffith.edu.au

By signing below, I confirm that I have read and understood the information package and in particular have noted that:

- I understand that my involvement in this research will involve providing a saliva sample from which a complete genome will be characterised;
- I understand that this study will not undertake any form of health testing;
- I understand that my DNA may be frozen for future use in this study;
- I agree the sample may be sent to members of the research team in other overseas centres for the purposes of this study;
- I have had any questions answered to my satisfaction;
- I understand the risks involved;
- I understand that there will be no direct benefit to me from my participation in this research;
- I understand that my participation in this research is voluntary;
- I understand that, because all samples have been de-identified prior to analysis, it is not possible to receive individual results;
- I understand that the information gained from this research may result in improved methods for analysis, but as an individual I do not have ownership of these results, the research records, or the sample that I give;
- I understand that if I have any additional questions I can contact the research team;
- I understand that I am free to withdraw at any time without explanation or penalty, in which case my DNA will be destroyed;
- I understand that I can contact the Manager, Research Ethics, at Griffith University Human Research Ethics Committee on (07) 3735 4375 (or research-ethics@griffith.edu.au) if I have any concerns about the ethical conduct of the project;

I agree to participate in the project.

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Appendix 7: Exemption from ethical review – ancient remains

Prof David Lambert
School of Natural Sciences
Nathan Campus
Griffith University

Confirmation of Exemption from Ethical Review and Approval
“Genetic Analysis of Non-Identified Ancient Human Remains”

I am pleased to confirm previous advice to you that, consistent with the standards and principles of the Australian National Statement on Ethical Conduct in Human Research (National Statement), research which involves only genetic analysis of non-identified ancient human remains is exempt from human research ethics review and approval.

For your information and future reference this advice is based on:
- Non-identified has the same meaning as Chapter 3.2 of the National Statement i.e. “data, which have never been labelled with individual identifiers … and by means of which no specific individual can be identified.”
- Section 13.0 of Booklet 30 (Research with Australian Indigenous Peoples) of the Griffith University Research Ethics Manual which outlines requirements for genetic research and retention for human tissues for studies involving Aboriginal or Torres Strait Islander people and clearly limits the scope of ethical review to biospecimens extracted from living people and cadaveric tissue.
- Chapter 3.5 of the National Statement which states that where “…research uses collections of non-identifiable data and involves negligible risk … [it] .. may therefore be exempted from ethical review.”

Please note; research that is exempt from ethical review must still be conducted in an ethical manner. Consistent with the advice in Chapter 4.7 of the National Statement, research involving genetic analysis of non-identified ancient Aboriginal or Torres Strait Islander remains should be conducted in accordance with the core values identified in the National Health and Medical Research Council guideline “Keeping research on track: A guide for Aboriginal and Torres Strait Islander peoples about health research ethics” and the principles and priorities set out in the “Guidelines for Ethical Research in Indigenous Studies” published by the Australian Institute of Aboriginal and Torres Strait Islander Studies.

Yours sincerely,

Rick Williams
Secretary, Griffith University Human Research Ethics Committee & Manager, Research Ethics & Integrity
Office for Research
Griffith University
Nathan QLD 4111
23 March 2017
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