Identification of individual koalas:

microsatellite analysis of faecal DNA

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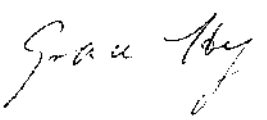
The Koala

Phascolarctos cinereus

Declaration

I hereby certify that the work contained in this thesis is my own, except where duly acknowledged, and that this work has not been submitted for a degree to any other University or similar institution.



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PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.

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Abstract

Current studies of koalas in the wild mainly rely on information gathered by traditional field methods, such as community sightings, spotlighting, radiotracking, animal trappings, ear tagging and faecal pellet incidence (Cork *et al.*, 2000; Curtin *et al.*,2002; Ward, 2002). Genetic sampling of individual koalas is difficult, as they are often located in tall trees in harsh terrain, such as steep, rugged gorges (Close, 1993). This is compounded by the size of an individuals homerange (1 to more than 200 hectares) (White, 1999; Cork *et al.*, 2000; Melzer, 2000; Ward 2002).

Collection of faeces is potentially the most reliable source of non-invasively obtaining DNA samples, which can be used to identify specific individuals (Gerloff *et al.*, 1994; Constable *et al.*, 1995, Wasser *et al.*, 1997). Direct observation of the animal providing the sample need not be made allowing extensive sampling of a single animal or a group of animals within a population to be carried out (Gerloff *et al.*, 1994; Waits and Leberg, 2000). Koala faecal pellets are easily distinguished from pellets of other animals in their homerange, by their shape and size.

This thesis demonstrates a simple, rapid and reproducible method of extracting DNA from koala faecal pellets using a commercially available DNA extraction kit, shows the maximum age of pellets from which DNA can reliably be extracted and defines the conditions required for the long term storage of pellets before DNA extraction is carried out.

Mitochondrial DNA PCR analysis provided a simple and rapid indication of the success of both the faecal DNA extraction and pellet collection process. Positive control tissue samples from known animals, amplified in conjunction with faecal DNA extracts, provides a visual reference of the success of each faecal DNA extraction. The faecal DNA was then successfully used for microsatellite analysis and the subsequent genetic profiling of individuals from within the Campbelltown koala population.

This study paves the way for the analysis of microsatellite loci in koala faecal pellet DNA to study populations, which are too sparsely distributed to allow the capture of individual koalas.

Abbreviations

A	Adenosine
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
bp	Base pairs
C	Cytosine
Ci	Curies
cm	Centimeters
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetra acetic acid
G	Guanine
H_E	Expected heterozygousity
H_O	Observed heterozygousity
IAM	Infinite allele model
KAM	K-Allele model
kb	Kilo base pairs
kg	Kilogram
M	Molar
mg	Milligram
min	Minutes
ml	Millilitres
mM	Millimolar
mtDNA	Mitochondrial DNA
ng	Nanogram
NSW	New South Wales
°C	Degrees Celsius
P	Phosphorus
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
s	Second/s
SMM	Stepwise mutation model
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TEMED	Tetra methylethylenediamine
µg	Microgram
µl	Microlitre
µM	Micromolar
V	Volts
v/v	Volume per volume
w/v	Weight per volume

1.0 Introduction

1.1 *Phascolarctos cinereus*

The family Phascolarctidae evolved approximately 15 million years ago (Martin and Handasyde, 1999), and *Phascolarctos cinereus*, more commonly known as the koala, is the only existing member of this family. The koala is an ecologically, physiologically and politically unique species and one of the most widely recognised mammals in the world (Cork *et al.*, 2000). It is endemic to Australia (Timms *et al.*, 1993; Martin and Handasyde, 1999) and colonies are widespread on the east coast of Australia, from northern Queensland to southwestern Victoria. They are seldom found in South Australia and never in Western Australia, the Northern Territory or Tasmania.

Koalas are arboreal, primarily nocturnal, and exist on a highly selective diet of Eucalyptus leaves (Ellis *et al.*, 1995). However, whilst many of the 200 species of Eucalyptus trees may house koalas, only a relatively small complement of these trees are used as a food source (White, 1999; Cork *et al.*, 2000; Phillips, 2000; Sluiter *et al.*, 2002). The koala is well adapted for its environment, having a well developed dental and digestive system, enabling it to cope with the high levels of allelochemicals and tannin, low nitrogen and high fibre content of the Eucalyptus leaves (Ellis *et al.*, 1995;White, 1999). Koalas have a low metabolic rate and therefore need to conserve energy. This is usually accomplished through periods of inactivity which last up to 18 hours per day and the use of low energy methods of preserving their homerange, e.g. scent marking and vocalisations (White, 1999). Koalas also possess rough pads on the palms and soles of their feet and have long sharp claws, which help them grip branches and trunks of trees. Their claws often mark the trees and these marks can be used to identify which trees koalas have visited, aiding the spotting of koalas in the wild (Martin and Handasyde, 1999).

Koalas weigh between 4-15 kg and can live up to 15 years in the wild (Cork *et al.*, 2000). They are generally solitary and reside within a home range usually no more than 3 hectares in size. However, homeranges from as little as 1 to more than 200 hectares have been reported (White, 1999; Cork *et al.*, 2000; Melzer, 2000; Ward 2002).

Homerange size varies due to social factors and habitat quality, the latter measured in terms of the density of preferred tree species. The sex of the animal also influences homerange size, as male koalas tend to occupy larger home ranges of lower quality habitat than females (White, 1999). Whilst this difference in homeranges may reflect differences in body size, it is usually because a dominant male has a homerange which overlaps that of several females, to which he maintains access during the breeding season (Lee and Martin, 1988; Cork *et al.*, 2000).

Breeding season lasts from October till May (Martin and Handasyde, 1999). In this time the male is highly active at night and constantly patrols his homerange, challenging and ejecting male rivals and mating with receptive females. The use of loud bellowing during the mating season also serves to announce the dominant male's presence and warn off male rivals (Martin and Handasyde, 1999).

The oestrous cycle of the koala is 27-30 days and the gestation period lasts up to 36 days (Martin and Handasyde, 1999). The female gives birth to a single infant, which migrates to the pouch where it will remain for up to 6 months. Once weaned after about 12 months, the young koala will often remain with its mother for a further 6 – 12 months (cited by Cork *et al.*, 2000), after which it will establish its own homerange. The males generally disperse whilst females usually remain close to their natal site (Martin and Handasyde, 1999; Fowler *et al.*, 2000).

Physical variation among koala populations, such as in adult size, muzzle shape, colour and thickness of fur, has led to the recognition of three subspecies. The subspecies, *Phascolarctos cinereus cinereus* in New South Wales, *Phascolarctos cinereus adustus* in Queensland and *Phascolarctos cinereus victor* in Victoria, are defined according to political borders rather than true genetic differences (Cocciolone and Timms, 1992; Worthington-Wilmer *et al.*, 1993; Houlden *et al.*, 1999; Melzer *et al.*, 2000; Sherwin *et al.*, 2000).

1.2 Trends in koala distribution and abundance

It is believed that koala populations, at the time of European settlement, were widespread but not abundant (Martin and Handasyde, 1999) and that displacement of Aborigines caused an increase in koala numbers. By the latter part of the nineteenth century, koalas were abundant enough for Europeans to take up koala hunting (Martin and Handasyde, 1999). This led to a dramatic decline in koala numbers throughout Australia, and the extinction of koalas in South Australia. Disease, deforestation and hunting caused a further abrupt decline in koala numbers in the early twentieth century.

During the 1920's, the decline in numbers prompted a program of koala relocation, which involved the transport of koalas from isolated populations on Phillip and French Islands to mainland Australia (Worthington-Wilmer *et al.*, 1993; Houlden *et al.*, 1996; Taylor *et al.*, 1997; Melzer *et al.*, 2000). This ongoing relocation program also temporarily alleviates the problem of deforestation on the Islands, due to overbrowsing (Houlden *et al*, 1996). Since 1923 the translocation of more than 10,000 animals to seventy different mainland sites in southern Australia (South Australia and Victoria) has occurred (Martin and Handasyde, 1999). Despite the initial numerical success of the program, population bottlenecks due to drought, bushfire, disease, over browsing, continuous relocations of individuals within populations and a reduction in habitat size, caused by logging and housing development, have continued to occur in southern Australian populations (Martin and Handasyde, 1999). However, in some areas of Victoria and on Phillip and Kangaroo Island in South Australia the problem of overpopulation remains (Melzer *et al.*, 2000).

It is thought that northern koala populations in New South Wales and Queensland have escaped severe bottlenecks. However, these populations have experienced a decline in numbers mainly due to natural disasters and habitat destruction through urban expansion (Lunney and Leary, 1988; Reed *et al.* 1990; Jurkis and Potter, 1997).

Today, koala colonies usually exist as isolated populations separated by areas of unsuitable habitat or cleared land (Martin and Handasyde, 1999). This makes it difficult to gauge accurately the size of individual populations. Determining true population numbers for the koala is compounded by the cryptic nature of the koala, as they are

often located in large trees in harsh terrain, such as steep, rugged gorges (Close, 1993). The few existing population statistics give inconsistent estimates and often fail to take into account a population's history and sociobiological factors, such as homerange fidelity and migration (Martin and Handasyde, 1999; Melzer *et al.*, 2000; Phillips, 2000). For example, the Australian Koala Foundation estimated the number of koalas throughout Australia to be between 45,000 and 80,000 (cited by Melzer *et al.*, 2000; Phillips, 2000). This contradicts the estimate of 75,000 – 130,000 koalas at Strathbogie Plateau in Victoria (cited by Melzer *et al.*, 2000; Phillips, 2000) and the National Parks and Wildlife Service of New South Wales (2001) estimate of 600,000 koalas in Queensland and 100,000 koalas in New South Wales.

Koala abundance also varies regionally, according to the availability of suitable habitat. For example, the abundance of koalas in local areas varies between less than 0.01 and 3 koalas per hectare in Queensland (Gordon *et al.*, 1990; White and Kunst, 1990; Melzer *et al.*, 2000), between 0.006 and 8 koalas per hectare in New South Wales (Gall, 1980; Gordon, *et al.*, 1990; Jurkis and Potter, 1997; Melzer *et al.*, 2000), between 0.7 and 8.9 koalas per hectare in South Australia (Melzer *et al.*, 2000) and up to 8 or 9 koalas per hectare in Victoria (Mitchell and Martin, 1990; Martin and Handasyde, 1999; Melzer *et al.*, 2000). The discrepancies in numbers are most likely due to differences in estimating numbers in local populations e.g. one researcher might calculate that every sighting in one area is equivalent to ten animals, while another may estimate 100 animals.

1.3 Introduction to Molecular Scatology

Obtaining information on wild animals has been a long-standing, logistical problem. Current studies of koalas in the wild mainly rely on information gathered by traditional field methods, such as community sightings, spotlighting, radiotracking, animal trappings, ear tagging and faecal pellet incidence (Cork *et al.*, 2000; Curtin *et al.*, 2002; Ward, 2002). When studying rare or enigmatic species, direct observation can be difficult and studying large populations relies on opportunistic sampling (Foran, *et al.*, 1997; Kohn and Wayne, 1997). In such cases, traditional methods can be expensive, time consuming and ineffective and it is rarely possible to monitor all individuals in a population (Haig, 1998; Mills *et al.*, 2000).

The advent of molecular genetic techniques offers new insights into conservation biology and behavioural ecology (Arnheim *et al.* 1990; Mills *et al.*, 2000; Sherwin *et al.*, 2000). However, genetic studies often rely on invasive methods of obtaining DNA samples, such as capturing individuals and taking blood or biopsy samples (Taberlet and Luikart, 1999; Taberlet *et al.*, 1999). These methods are sometimes difficult, unsafe and stressful for both the animal and handler (Constable *et al.*, 1995; Kohn *et al.*, 1995; Kohn and Wayne, 1997; Levy, 1999). Therefore a method of noninvasively obtaining DNA samples, which can be used to identify specific individuals, is required.

Collection of faeces is potentially the most reliable source of obtaining these DNA samples (Gerloff *et al.*, 1994; Constable *et al.*, 1995, Wasser *et al.*, 1997). Direct observation of the animal providing the sample need not be made and the collection and transport of faeces does not require approval from an animal ethics committee. This allows extensive sampling of a single animal or a group of animals within a population without disrupting the animals' normal behaviour (Gerloff *et al.*, 1994; Waits and Leberg, 2000). Koala faecal pellets are easily distinguished from pellets of other animals in their homerange, by their shape and size (see figure 2.1). Faecal pellets are already used to indicate koala presence (Allen, 2000) and pellet distribution studies can be used in conjunction with radiotracking to investigate diet selection and habitat use (Ellis *et al.*, 1998; Phillips and Callaghan, 2000; Sluiter *et al.*, 2002).

Molecular scatology has been useful for assessing genetic structure, reproductive status, habitat use and homerange of a variety of mammals including the brown bear and dugong (Kohn and Wayne, 1997), seals (Reed *et al.*, 1997), chimpanzees (Constable *et al.*, 2001) and wombats (Banks *et al.*, 2002). This process detects variations in specific regions of DNA, which are passed from parent to offspring (i.e. inherited variations). These variations can be unique to individuals and serve as genetic markers.

1.4 Genetic markers

Initially, genetic variation in animal populations was detected through the analysis of protein polymorphisms, such as allozymes, which show differential migration in an electric field (Lewontin and Hubby, 1966; Avise, 1994; Engel *et al.*, 1996; Houlden *et al.*, 1999). However, only approximately one third of base substitutions in the DNA result in amino acid changes and only about 25% of base substitutions produce changes in protein structure which can be detected by electrophoresis (Baker, 2000). Therefore the total number of alleles detected at a particular locus by allozyme electrophoresis may not reflect the total genetic variation at that locus. The low level of variability among allozymes may mask heterogeneity in some organisms, which is required for the analysis of population structure and phylogenetic studies (Hughes and Queller, 1993; Taylor *et al.*, 1994; Engel *et al.*, 1996; Haig, 1998, Baker, 2000).

The advent of the polymerase chain reaction (PCR) in the early 1980's, and the discovery of hypervariable DNA loci, such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs), mitochondrial DNA (mtDNA) sequence variants, as well as minisatellites and microsatellites, has revolutionised our ability to predict accurately the amount of genetic variation within a particular species (Engel *et al.*, 1996; Haig, 1998; Waits *et al.*, 2000). These markers detect a higher level of genetic variation than allozymes, making them superior for genetic population studies (Bruford and Wayne, 1993; Hughes and Queller, 1993; Engel *et al.*, 1996).

However, the nature of a study and its hypotheses influence the choice of genetic marker. This study proposes that individual koalas may be identified from faecal DNA. It therefore requires genetic markers that will be able to amplify partially degraded DNA and that can detect the highest level of variation possible. MtDNA sequence variants and microsatellites are ideal markers for these tasks as they can be used on partially degraded DNA, they produce patterns which are easy to interpret after electrophoresis and have previously been used for population and genetic studies of the koala (Houlden *et al.*, 1996b; Houlden *et al.*, 1999; Fowler *et al.*, 2000).

1.5 Mitochondrial DNA

Animal mitochondrial DNA is a small, circular molecule (approximately 16,000 bases long), lacking recombination, repetitive DNA, spacers and introns. It encodes various RNA molecules and proteins (Randell, 1991; Randi, 2000) and occurs in multiple copies in each cell (Kohn *et al.*, 1995; Zhang and Hewitt, 1996; Wayne *et al.*, 1999; Randi, 2000).

The main non-coding sequence of mtDNA is in the control region (called the D-loop in vertebrates), which regulates the replication and transcription of the entire mitochondrial genome (Randi, 2000). This control region has a high rate of evolution, due to base substitution, which increases the effects of random genetic drift and provides a high rate of genetic differentiation (Brown, 1985; Moritz, 1994; Taylor *et al.*, 1997; Houlden *et al.*, 1999; Fowler *et al.*, 2000). Analysis of the variation of the mtDNA control region has proven to be an asset for the study of phylogenetic and phylogeographic relationships and has resolved population structure and gene flow patterns among a large number of different taxa, including that of the koala (Taylor *et al.*, 1997; Houlden *et al.*, 1999; Fowler *et al.*, 2000; M.E. Montgomery, pers comm), shark, alligator and human (Randi, 2000).

Due to its high copy number per cell, mtDNA is likely to be present in greater quantities than nuclear DNA (Kohn *et al.*, 1995; Reed *et al.*, 2000). This makes mtDNA a potentially useful tool to test the suitability of faecal DNA in the polymerase chain reaction (PCR) (Reed *et al.*, 2000). However, due to its maternal mode of transmission, mtDNA can only provide information about female-mediated gene flow. Therefore, the characterisation of the genetic status of mammals using nuclear DNA markers is essential. Recent studies (Moritz, *et al.*, 1997; Reed *et al.*, 1997; Waits *et al.*, 2000) have proven that a combination of mtDNA markers and nuclear microsatellites is a powerful tool for the analysis of genetic population structure in mammals.

1.6 Microsatellite Markers

Microsatellite loci are widely dispersed within the eukaryotic genome (O'Connell *et al.*, 1996). They are found, on average, every 10kb in eukaryotes (Tautz, 1989) and consist of short (2 - 6 base pairs) motifs repeated in tandem. Microsatellites are composed of pure (or perfect) (e.g. CAG_{10}), compound (e.g. CAG_3CTG_7) or imperfect (e.g. $CAG_3TTCA_4TTCA_2$) sequences (Engel *et al.*, 1996; Jarne and Lagoda, 1996; Rosenbaum and Deinard, 1998; Schlotterer and Pemberton, 1998), which are flanked by unique conserved sequences specific to each locus (Tautz, 1989; Buchanan, 1996). Microsatellite loci are highly polymorphic due to variations in the number of repeat units in a series. This variation commonly allows a single locus to possess a large number of alleles (Taylor *et al.*, 1994; Engel *et al.*, 1996; Jarne and Lagoda, 1996), and means that microsatellites are more frequently heterozygous than other genetic markers (Hughes and Queller, 1993; Taylor *et al.*, 1994). The most common repeat motif of microsatellites is $(CA)_n$/ $(GT)_n$ (Buchanan *et al.*, 1996). Microsatellites are commonly present in non- coding regions (introns) of DNA and only rarely found in coding regions (exons), where changes in the number of repeat units in an array can cause severe illness, such as Huntington's disease (Zuhlke *et al.*, 1993).

Microsatellites are an ideal genetic marker, due to their codominant Mendelian inheritance, high polymorphism and selectively neutral evolution (Bruford and Wayne, 1993; Jarne and Lagoda, 1996). Their small size, generally less than 300 base pairs (Taylor *et al.*, 1994), makes them suitable for amplification using PCR. The polymorphisms are detected using primers designed to detect the unique flanking sequences of the repeat motif. The amplified PCR products (alleles) can then be identified on polyacrylamide DNA sequencing gels (Schlotterer and Pemberton, 1998), where the alleles at each microsatellite locus appear as either one (homozygote) or two (heterozygote) bands (Tautz, 1989; Blouin *et al.*, 1996; Engel *et al.*, 1996). The size of these bands can be determined by comparison to a DNA size marker, run in conjunction with the samples.

Amplification of microsatellite loci often produces stutter bands, also called shadow bands, surrounding the authentic allele. These artifacts are thought to be caused by slippage of the *Taq* DNA polymerase during the first few cycles of PCR, when single

repeat units are inserted or deleted from the microsatellite array (Tautz, 1989; Taberlet *et al.*, 1996; Schlotterer and Pemberton, 1998). Stutter bands can often hinder the analysis of microsatellite loci, especially when allele bands overlap (Scribner and Pearce, 2000) or when the stutter bands have an equal intensity to the alleles (Queller *et al.*, 1993). The intensity of stutter bands can be reduced and their potential for error reduced, by adjusting the annealing temperature, decreasing the number of PCR cycles, or decreasing the amount of *Taq* polymerase (Scribner and Pearce, 2000). However, these steps will also reduce the sensitivity of the reaction and may hinder the detection of the microsatellite locus.

Repeat sequences in the DNA are subject to a higher degree of mutation than single copy DNA, thus providing a source of variation for genetic population studies (Avise, 1994). Variation in microsatellite loci has also been attributed to slippage of DNA polymerase during DNA replication, as well as unequal meiotic exchange (Levinson and Gutman, 1987; Engel *et al.*, 1996; Jarne and Lagoda, 1996; Scribner and Pearce, 2000). These mutational mechanisms vary the length of microsatellites, resulting in the gain or loss of one repeat, or in the change of multiple repeats in the array. Variation to the flanking regions of microsatellites, especially to the primer binding site, or deletion of loci, may produce a null allele (also called non amplifying alleles) (Paetkau and Strobeck, 1995; Engel *et al.*, 1996; Jarne and Lagoda, 1996). Null alleles prevent primers from annealing and appear as a failed PCR reaction when homozygous, or as one allele when heterozygous (Taberlet *et al.*, 1999; Waits *et al.*, 2000). Null alleles can cause errors in the calculation of population statistics and may lead to erroneous rejection of the Hardy- Weinburg equilibrium, due to the presence of excess homozygotes (Engel *et al.*, 1996; Jarne and Lagoda, 1996). Therefore all data should be screened for null alleles during analysis. This may be done by testing the frequency of mutations to the primer annealing sites against frequencies that are expected under the Hardy Weinburg equilibrium (Paetkau and Stroebeck, 1995), or by comparing the inheritance pattern of parental alleles in offspring (Callen *et al.*, 1993). The latter method of screening for null alleles was used in the present study, as the relatedness of many of the koalas sampled was known.

The rate of mutation affects the level of variation observed in a population, and this rate can vary considerably between different taxa and loci. Mutation rates range from (on average) 1.2×10^{-3} spontaneous mutations per locus per gamete for humans to as low as 10^{-6} for *Drosophila* (Weber and Wong, 1993; Buchanan *et al.*, 1996; Taylor *et al.*, 1997; Schug *et al.*, 1997).

Three mutational models have been proposed to explain the mutation process of microsatellites. The first, the infinite allele model (IAM) (Kimura and Crow, 1964; Jarne and Lagoda, 1996; Charlesworth, 1998; Rosenbaum and Deinard, 1998), states that single mutations will produce new alleles not previously found in the population, thus providing an infinite number of allelic states. The second model, the stepwise mutational model (SMM), proposes the generation of a new allelic state by a single mutational event, resulting in an increase or decrease in the number of repeat units in an array by one repeat unit (Kimura and Ohta, 1978; Jarne and Lagoda, 1996; Rosenbaum and Deinard, 1998). The third model, the K allele model (KAM), is described by Di Rienzo *et al.* (1994) as an intermediate between the IAM and SMM and appears to be the most accurate model for describing microsatellite evolution at the population level. In the K model, most mutations are single step mutations, but large jumps in repeat unit numbers also occur i.e., K allelic states are possible (Jarne and Lagoda, 1996; Rosenbaum and Deinard, 1998).

Homoplasy, a consequence of mutation, is the co-occurrence of alleles which are identical in state but are not identical by descent i.e. they arrive at the same size through convergent evolution (Jarne and Lagoda, 1996; Scribner and Pearce, 2000). Even though homoplasy is not seen to be of concern in population studies, disregarding homoplasy may lead to an underestimation of the actual amount of divergence between populations (Jarne and Lagoda, 1996; Scribner and Pearce, 2000). Homoplasy is easily detected by comparing the DNA base sequence of alleles of the same size, as homoplasy appears as a variation in the microsatellite flanking sequence (Jarne and Lagoda, 1996). My study examines differences within populations; therefore it is not essential to screen for homoplasy. However, screening for homoplasy may be required when comparing koala populations from different regions of Australia.

Microsatellite markers are the most commonly used genetic markers for parentage studies and are an excellent tool for linkage mapping, phylogenetic studies, forensics and studying animal homeranges (Jarne and Lagoda, 1996). Microsatellites can be amplified from nanogram quantities of DNA and from partially degraded DNA (Taylor *et al.*, 1994; Ishibashi *et al.*, 1995). This versatility makes them ideal for use in molecular scatology, as nuclear DNA in faeces is usually present in low quantities and is degraded (Constable *et al.*, 1995; Kohn and Wayne, 1997). Microsatellites have also been used to amplify DNA from museum specimens (Taylor *et al.*, 1994; Moritz, 1994), road kills (Heckenburg, 1997), skin (Richard *et al.*, 1996) and urine (Valarie and Taberlet, 2000). Microsatellites can be amplified from fresh and old tissue samples, proving they are a versatile tool for genetic studies.

1.7 Extraction of DNA from Faeces

Cells shed from the intestinal lining of the koala adhere to the outer mucosal layer of the faeces as they pass through the intestines. Once the pellets are collected, these cells can be separated from the faeces and the DNA extracted. Although many techniques have been described for the extraction of DNA from faeces, Flagstad *et al.* (1999) found that the most crucial step in any faecal DNA extraction is the initial surface wash of the faeces. The wash should produce a 'clean' supernatant containing as many cells as possible (Flagstad *et al.*, 1999). Multiple washes and DNA extractions should be carried out on the one pellet and pooled to optimise results (Kohn *et al.*, 1995). Only a small number of cells adhere to each pellet; consequently, only minute quantities of DNA are extracted from the faeces (Kohn and Wayne, 1997; Flagstad *et al.*, 1999; Taberlet *et al.*, 1999).

With any faecal PCR there can be technical problems which can lead to genotyping errors and incorrect results. Three problems are significant when utilising faecal DNA. The first is the inhibition of *Taq* polymerase during PCR by substances in the faeces (e.g. micro-organisms, degradative enzymes, mucus or plant polysaccharides) (Sidransky *et al.*, 1992; Deuter *et al*, 1995; Kohn and Wayne, 1997; Taberlet *et al.*, 1999). This problem is easily overcome by using an inhibitor removal complex such as bovine serum albumin or a polysaccharide matrix, which is added to the early stages of DNA extraction.

Secondly, the age of the pellets is difficult to estimate as faecal samples often remain in the field for several weeks before collection. This promotes the degradation of DNA via exposure to U.V. light, insects and extreme weather conditions (Carter, 2000). The use of this degraded DNA results in genotyping errors such as the production of false alleles or allelic dropout. False alleles occur when PCR artefacts (e.g. stutter bands or contaminating DNA) are preferentially amplified over template DNA, whilst allelic dropout is a result of stochastic sampling in which only one of two alleles is amplified at a heterozygous locus (Taberlet *et al.*, 1996; Kohn and Wayne, 1997; Taberlet *et al.*, 1999; Morin 2001). A multiple-tube approach to faecal PCR can reduce mistyping errors. This consists of repeating PCR experiments 3-7 times, using aliquots of the same extract (Navidi *et al.*, 1992; Taberlet *et al.*, 1996; Kohn and Wayne, 1997; Taberlet *et al.*, 1999; Waits and Leberg 2000; Morin *et al.*, 2001). A multiple-tube approach will be used in my study to ensure the correct genotypes are obtained for all koala faecal DNA samples. A method of preserving faecal DNA to prevent degradation prior to extraction will also be examined.

The third of these significant problems is sample contamination. Contaminating DNA may be amplified during PCR and cause misleading results. Sample contamination can occur both in the field and in the laboratory at any stage of sample processing. Contamination in the field occurs prior to or during sample collection as a result of fungal or bacterial infection, insects and larvae infestation, human contact, or by cross-contamination from other pellets (Hummel and Herrmann, 1994, Foran *et al.* 1997). Although bacterial contamination is unavoidable on faecal samples, the risk of other contaminants affecting the samples in the field can be minimized by only collecting intact pellets, using a preservative to prevent bacterial, fungal growth and degradation of DNA, wearing disposable gloves to collect each sample and by collecting individual samples in clean containers (e.g. envelopes, snap-lock bags etc).

Sample contamination in the laboratory is most likely to be introduced during DNA extraction via contaminated reagents or by co-purification of another DNA source (e.g. skin cell, plant matter in koala faeces), or during PCR set up via carry-over products i.e. the transfer of amplified products of former reactions to the new reaction by indirect (e.g. aerosols) or direct contact (e.g. gloves) (Hummel and Herrmann, 1994). Reagent

contamination can be monitored using negative controls. The two commonly used negative controls are an extraction control and a non template control. The extraction control undergoes all extraction, purification and amplification procedures, but does not contain any sample material consequently it reveals minor contaminants in the DNA extraction reagents (Hummel and Herrmann, 1994). The non template control is set up at the same time as each PCR reaction and contains all PCR reagents except DNA, thus revealing minor contaminants in the reagents used for PCR(Hummel and Herrmann, 1994). Sample contamination can be avoided by having separate work areas for PCR set up and DNA extraction and using aerosol resistant pipette tips (Taberlet *et al.*, 1996; 1999; Kohn and Wayne, 1997).

Successful molecular scatology studies using mtDNA and microsatellite markers have been conducted on European Brown Bears (*Ursus arctos*) (Hoss *et al.*, 1992; Kohn *et al.* 1995; Wasser *et al.*, 1997), baboons (*Papio cynocephalus*) (Constable *et al.*, 1995), bonobos (*Pan paniscus*) (Gerloff *et al.*, 1995), dugong (*Dugong dugong*) (Tikel *et al.*, 1996), polar bears (*Ursus maritimus*) (Khon *et al.*, 1995), giant pandas (*Ailuropoda melanoleuca*) (Ding *et al.*, 1998), seals (*Halichoerus grypus and Phoca vitulina*) (Reed *et al*, 1997), mountain lions (*Puma concolor*) (Ernest et al., 2000) and wombats (*Vombatus ursinus*) (Banks *et al*, 2002). Clarke (2000) has also successfully amplified DNA from koala faecal pellets in a pilot study.

1.8 Current studies of the Koala

Severe population bottlenecks in the early 1930's and the subsequent translocation of koalas from island populations, originally founded by 2-3 individuals (cited by Houlden *et al.*, 1999), produced a founder effect on allelic diversity within southern koala populations i.e. it created mainland populations with a similar genetic diversity to the island populations (Taylor *et al.*, 1997; Houlden *et al.*, 1999). This implies that southern koala populations possess a low level of genetic diversity.

Minisatellite and mtDNA-RFLP analyses (Taylor *et al.*, 1991; Taylor et *al.*, 1997; Houlden *et al.*, 1999; Fowler *at al.*, 2000; Seymour *et al.*, 2001) conducted on koala populations throughout Victoria and South Australia, have verified that the southern koala populations exhibit low genetic variation. In contrast, northern koala populations

(New South Wales and Queensland), which have escaped major population disturbances, retain a greater degree of genetic diversity (Taylor *et al.*, 1997; Houlden *et al.*, 1999; Fowler *et al.*, 2000).

The low level of genetic diversity in the southern populations and the understanding that microsatellites could detect a greater level of genetic diversity at a single locus than other methods prompted Houlden *et al.* (1996a) to develop six microsatellite primer sets for use in the koala. The polymorphic nature and Mendelian inheritance of the amplified microsatellites was revealed in a paternity exclusion study on captive koalas (Houlden *et al.*, 1996a). These six loci have since been used to assess the effect of a road barrier on gene flow between adjacent koala colonies (Brown, 2000) and to determine the level of genetic variation and genetic differentiation in south-eastern and north-eastern koala populations (Houlden *et al.*, 1999; Seymour *et al.*, 2001). The latter studies confirm previous findings that there is less genetic diversity in southern koala populations than northern populations.

This thesis analyses koala populations in southern Sydney regions of NSW (Figure 1.1), primarily in the Campbelltown area. Since the late 1980's the Campbelltown population has been monitored and studied using community sightings, ear tagging, radio tracking and scat analysis (Close 1993; Sluiter *et al.*, 2002; Ward, 2002). The population consists of between 90-200 individuals (Ward, 2002) and many familial relationships within this population are known (Close, 1993; Ward and Close, 1998; Ward, 2002). The ongoing capture and tagging of Campbelltown koalas has provided tissue samples for many individuals in this population and allowed Dr. B.A. Houlden to establish a preliminary genetic database for 43 individuals in 1998, using her six koala microsatellite loci (see Appendix 4). The average heterozygosity of this population for forty koalas is 0.607 (Appendix 4), which is lower than north-eastern populations (av. H_E= 0.851) and greater than southern populations (av. H_E= 0.436) (Houlden *et al.*, 1999, Sherwin *et al.*, 2000), indicating that the Campbelltown population has not suffered severe population disturbances and retains a moderate level of genetic variation, comparable to other wild animal populations (O'Connell *et al.*, 1996; Moritz *et al.*, 1997; Banks *et al.*, 2001).

The genetic profiles obtained by Houlden for most individuals sampled from the Campbelltown population differ from each other at one, two or three loci. However, two pairs of individuals, CM3 and CM16, and CM11 and CM20, possessed identical profiles. Capture records indicate that the DNA samples for each pair were not obtained from the same individuals. This demonstrates that the six known loci (Houlden *et al.*, 1996a) do not detect enough variation within the Campbelltown population to clearly differentiate between individuals, illustrating the need for additional loci. Recently, whilst investigating the mating system of koalas, PhD student Valma Cahill (Macquarie University) isolated twenty-two new koala microsatellite primers (Cahill, 2001). Twelve of these polymorphic primers have been obtained for evaluation in the present study. Successful primer sets will be used to expand B.A. Houlden's preliminary genetic database for the Campbelltown population and ensure that a unique genetic profile can be obtained for all individuals.

Clarke's (2000) pilot study showed that two primers of Houlden *et al.* (1996) could amplify DNA obtained from the surface of koala faecal pellets. Though Clarke (2000) experienced some difficulty with the PCR reactions, most likely due to inhibition of the PCR by faecal substances in the DNA extraction (Kohn and Wayne, 1997), he amplified both mtDNA and microsatellite DNA. Comparison to a positive control, in this case a koala tissue sample, confirmed that the loci being amplified belonged to the koala from which the faecal DNA had been extracted. Clarke (2000) also showed that it was possible to obtain DNA from koala faecal pellets which had been stored for two months at room temperature. This is a positive discovery, as it is often not possible to collect fresh pellets or conduct molecular studies of samples immediately after obtaining them in the field. It also allows collections to be made in the field without specialised equipment and dispatched to the laboratory.

This study expands Clarke's preliminary findings and aims to perfect a simple, rapid and reproducible method of extracting DNA from koala faecal pellets, determine the maximum age of pellets from which DNA can reliably be extracted and examine the conditions required for the long term storage of pellets before DNA extraction is carried out. All faecal DNA extracts will also be analysed using heterologous mitochondrial primers (Houlden *et al.*, 1999), to amplify a portion of the control region of mtDNA. A

comparison of the faecal results to a koala tissue sample will be used to determine the species of origin of the faecal DNA samples. The faecal DNA will then be used for microsatellite analysis and the subsequent genetic profiles used to identify individuals from within the Campbelltown koala population. If successful, the analysis of microsatellite loci in koala faecal pellet DNA could be used to study populations which are too sparsely distributed to allow the capture of individual koalas.

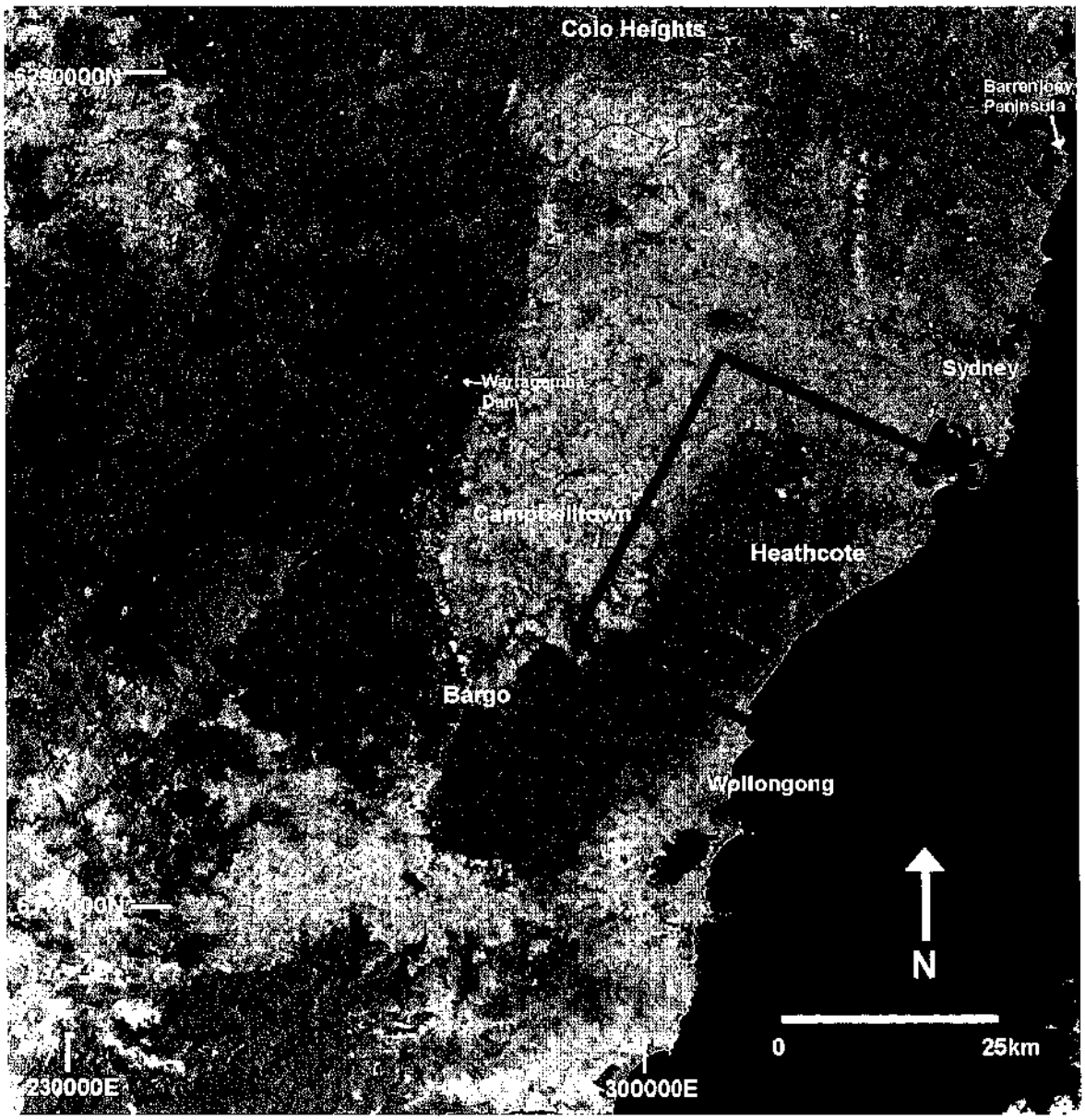


Figure 1.1 Map of New South Wales highlighting the koala populations sampled during this study. ● -Indicates the positions of the koala populations of Campbelltown, Heathcote, Bargo and Colo Heights. The southern Sydney region of NSW is also shown. Maps and information courtesy of Ward, (2002) and Geoscience Australia, Canberra. Crown Copyright ©. All rights reserved. www.ga.gov.au

1.9 Aims and Objectives

The specific aim of this study was to develop a method of identifying individual koalas from faecal DNA. This was accomplished by:

(i). developing a simple protocol for the collection of koala faecal pellets which can be used for DNA extraction.

(ii). developing and refining a standard method of extracting DNA from old and fresh faecal pellets.

(iii). determining the optimal storage time and conditions for preserving DNA on faecal pellets prior to DNA extraction.

(iv). conducting mtDNA analysis of faecal DNA using tissue samples as a positive control.

(v). optimising PCR conditions for six known and twelve new koala microsatellite primers

(vi). choosing suitable koala microsatellite primers for faecal DNA analysis, which provide a unique genetic profile for individuals.

(vii). conducting a blind trial of methods using faecal DNA extracted from known Campbelltown koalas during this study.

2.0 Methods

2.1 Field Work

2.1.1 Sample collection.

During weekly tracking expeditions, Associate Professor R.L. Close, Dr. S. Ward, Mrs L. Coxall and Mrs Marylin Jones collected pellets from the base of trees containing known adult and juvenile koalas in the southern Sydney population at Campbelltown, NSW. The ages of the pellets were assessed on site. Pellets estimated as less than five weeks old and possessing a patina (a shiny layer of mucus) were collected. The pellets were identified as koala pellets by their size and shape (Figure 2.1). Ear punch tissue or blood samples and pellets were also collected from animal trapping procedures. Where possible, ear punches and pellet samples from previously unidentified koalas were also collected.

The pellets were allowed to air dry and then stored at 4°C. Tissue samples were stored at -70°C.

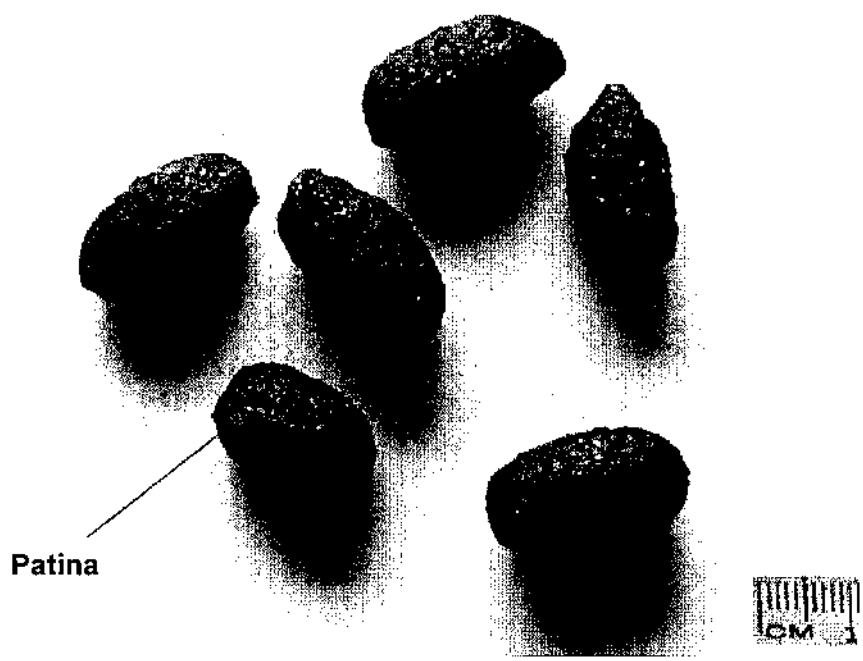


Figure 2.1. Appearance of koala faecal pellets collected for DNA extractions. Note the patina on all the pellets.

2.2 Laboratory procedures

2.2.1 DNA extraction from tissue

Purification of koala genomic DNA was carried out using the DNeasy Tissue Kit (Qiagen Pty, Ltd). Buffers and spin columns used in the extraction were supplied in the kit. Approximately 10 mg of ear punch tissue, or up to 25 mg of other tissue was incubated with 180 µl of Buffer ATL and 20 µl proteinase K (20mg/ ml) at 55°C until the tissue was completely lysed. Following incubation, 4 µl of RNase A (DNase-free, 100mg/ml) was added, to degrade cellular RNA, and the solution incubated for a further 2 min at room temperature. Buffer AL (200 µl) was then added to the cleared lysate and the solution incubated at 70°C for 10 min. The DNA was precipitated at room temperature with 200 µl absolute ethanol and transferred to a DNeasy spin column, containing a silica-gel membrane. The DNA was bound to the membrane by centrifugation at 6000 g. The column was then washed via centrifugation with 500 µl of buffer AW1 followed by 500 µl of buffer AW2. An additional centrifugation at 6000 g for 3 min removed residual buffer. The DNA was eluted from the column, in 100 µl of buffer AE, by centrifugation at 6000 g for 1 min. To increase DNA yield, this elution step was repeated and the eluates pooled. Extracted genomic DNA samples were stored at -20°C until required.

2.2.2 Faecal DNA extraction

Individual pellets were placed in small, sterile plastic bags with 400 µl of phosphate-buffered saline (PBS; 50 mM $NaH_2PO_4.2H_2O$, 50 mM Na_2HPO_4, 0.15M NaCl, pH 7.4) and the surface of the pellet was gently washed to release the shed epithelial cells of the intestinal lining. Disintegration of the pellet was carefully avoided by washing the pellet for only 1 min. 200 µl of the supernatant was removed and placed in a 2 ml microcentrifuge tube. A further 200 µl of PBS buffer was added to the pellet and gentle agitation applied. This 200 µl was removed and placed in a second 2 ml microcentrifuge tube ready for DNA extraction using a QIAamp® DNA mini stool kit (QiagenPty, Ltd). Buffers and spin columns used in the extraction were supplied in the kit.

To ensure complete lysis of all cellular material, 1.6 ml of buffer ASL was added to both 200 µl extracts. Incubation times of 5, 10, 20, 30, 45 and 60 min at 35°C were assessed and the optimal time of 45- 60 min used for subsequent extractions. During incubation with buffer ASL, samples were occasionally vortex mixed to maintain a homogeneous mixture. Following incubation, pellet debris was removed from the lysate via centrifugation and 1.4 ml of the supernatant transferred to a new 2 ml microcentrifuge tube. One inhibitEX tablet was added to each lysate and dissolved by vortex mixing. Standing the lysate at room temperature for one minute allowed DNA-damaging substances and PCR inhibitors to be absorbed in to the inhibitEX matrix. The inhibitors were then removed by centrifugation at 13,000 g for five minutes. The supernatant (~ 800 µl) was removed, placed in a sterile 1.5 ml microcentrifuge tube and centrifuged for a further 5 min, to ensure the removal of all inhibitors. 600 µl of the supernatant was then transferred to a new 2 ml tube containing 25 µl proteinase K (20mg/ ml) and 600µl of Buffer AL was added. The mixture was vortexed to form a homogeneous solution and incubated at 70°C for 10 min. The DNA was precipitated at room temperature with 600µl of absolute ethanol. 600µl of the first lysate from each pellet was transferred to a DNeasy spin column, containing a silica-gel membrane. The DNA was bound to the membrane by centrifugation at 13,000 g. This was repeated until both the extracts from the same pellet had passed through the one column. The column was then washed by centrifugation with 500 µl of buffer AW1 followed by 500 µl of buffer AW2. The column was centrifuged for a further 3 min at 13,000g to remove residual buffer. DNA was eluted by centrifugation at 13,000 g for 1 min after incubation for 5 min in 100 µl of buffer AE at room temperature. To increase DNA yield, the elution step was repeated using 60 µl of buffer AE and the eluates pooled. Extracted pellet DNA samples were stored at -20°C.

An extraction containing 200 µl PBS and no faces was also performed, to detect whether contamination had occurred during the extraction procedure (extraction negative control).

2.2.3 Estimation of genomic and pellet DNA quantity and quality

To estimate the quantity and quality of the DNA, electrophoresis of the samples was carried out on agarose gels (2%, Seakem) at 95V. Tris-acetate-EDTA buffer (TAE; 0.04 M Tris base, 0.04 M acetate, 0.001 M EDTA, pH 8.0) was used in the preparation and running of the gels. 2.5 µl of loading buffer (15% (w/v) Ficoll-4000, 0.25% (w/v) bromophenol blue and 5mM EDT, pH 7.5) was added to a 20 µl aliquot of DNA. DNA samples (22.5 µl) were then loaded onto the gel and electrophoresis begun. Electrophoresis was stopped when the loading buffer had migrated approximately ¾ the length of the gel or after 45-60 min. DNA was stained with ethidium bromide (5µg ml^{-1}) and visualised with ultra violet light using a transilluminator.

The quantity of DNA in each sample was estimated by comparing band intensity to an SPP-1/ Eco R1 marker (Promega) run in parallel to the samples. Both the size and concentration of each marker band is given in Appendix 1. A photograph of the gel was taken using a CU-5 Polaroid camera with Wratten 23A filter and type 667 film (Polariod Corporation Pty Ltd).

2.2.4 Spiked pellet experiment

To verify the procedure in section 2.2.2, DNA was extracted from frozen liver tissue of a water rat (*Hydromys chrysogaster*) using the method described in section 2.2.1. Ten nanograms of this DNA was air dried onto the surface of two old koala faecal pellets. Once dry the DNA was extracted from the surface of the pellets and microsatellite analysis of the tissue and faecal DNA conducted using water rat locus W2A1 (PCR primers: F-5'GGCTATGTCATGGGAAGGC 3'; R-5'AGAGGATCCCATAAGCTGACAC 3') and the reaction conditions determined by Hinds (1999). Microsatellites were analysed using the procedures in section 2.2.9 - 2.2.14 and the following touchdown PCR program:

An initial one cycle denaturation of 94°C for 4 min, was followed by 5 cycles of 94°C for 1 min, 65°C for 50 s, 72°C for 1 min, with the annealing temperature decreasing by 2°C per five cycles, followed by 15 cycles using an annealing temperature of 57°C. The

reaction was finalised with a single cycle of 94°C for 30 s, 55°C for 30 s, 72°C for 4 min.

2.2.5 Mitochondrial DNA analysis of faecal DNA

MtDNA analysis was used to confirm that faecal DNA was koala DNA. Mitochondrial DNA was amplified in thin walled 0.5ml PCR tubes (Boheringer Manheim, Pty. Ltd) using an OMN-E U well thermal cycler (Integrated Sciences). The reaction conditions were selected based on advice from Dr. M. Campbell (U.W.S) and Mrs Marylin Jones (U.Syd.).

Mitochondrial primers 15996L (5' TCCACCATCAGCACCCAAAGC 3') and 16502H (5' TTTGATGGCCCTGAAGTAAGAACCA 3') (cited in Houlden *et al.*, 1999) were synthesized by Gensetoligos (Proligo Australia Pty, Ltd) and stored at -20°C. The primers were diluted to a working concentration of 20μM. Each reaction consisted of 1x Mg free Thermophilic buffer [Integrated Sciences, Pty, Ltd. or Astral Scientific Pty, Ltd.], 2.5 mM $MgCl_2$ [Integrated Sciences, Pty, Ltd. or Astral Scientific Pty, Ltd.], 0.5 U *Taq* DNA polymerase [Integrated Sciences, Pty, Ltd.] or 0.5 U BioTAQ DNA polymerase [Astral Scientific Pty, Ltd.], 200 μM of dGTP, dCTP, dTTP and dATP [Promega], and 10 μM each desalted primer [Gensetoligos]. One or two microlitres of pellet DNA was added to each reaction, giving a total volume of 1 μl. A positive control containing 1 μl of tissue DNA in a final volume of 10 μl and a negative control were also set up. Aerosol resistant pipette tips (Molecular Bio-Products) were used in all material transfers and, to prevent evaporation, all reactions were overlaid with 25 μl of liquid paraffin (Fauldings). PCR tubes were transferred to an OMN-E U well thermal cycler (Integrated Sciences) and the following program run:

An initial one cycle denaturation of 94°C for 3 min, was followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final cycle of 94°C for 30 s, 55°C for 30 s, 72°C for 3 min.

Following PCR, 8 µl of each reaction was placed in a 1.5ml microcentrifuge tube and 2.5 ul of loading buffer added before loading the products onto a 2% agarose gel. Electrophoresis was carried out as described in section 2.2.3.

2.2.6 Faecal DNA preservation studies

The storage of pellets at 4°C was examined as a short term measure for preserving pellet DNA before extraction and analysis. Pellets were taken directly from the colon of a koala, which had been killed by a car, and allowed to air dry for approximately one hour. Immediately after drying, DNA was extracted from the surface of two pellets using the procedure in section 2.2.2. The remaining pellets were stored at 4°C and extractions carried out every month for a period of six months. A DNA extraction was also carried out on two pellets stored for six months at -70°C.

A method for the long term storage and preservation of pellet DNA at room temperature was also examined. UWS undergraduate Advanced Science students, Daniel Obando and Emily Daly, carried out this work under my supervision, using approximately forty pellets collected from the colon of a road kill koala.

To determine whether DNA is lost when wet pellets are wrapped in paper during collection, five pellets were taken and placed onto one of the following paper types: paper towel, tissue, filter paper from a slide box and Whatman 1mm filter paper. All forty pellets were then allowed to dry in the wind and sun for approximately one hour. After drying, DNA was extracted from the surface of these pellets and from the papers. The paper extracts were then compared to the pellet extracts, using the methods in sections 2.2.3 and 2.2.5.

The remaining pellets were separated into two groups. One group was sprayed four times with a fine mist of 70% ethanol to lightly coat the pellet. The second group was not sprayed. Immediately after spraying, DNA was extracted from two pellets from the both groups, using the procedure in section 2.2.2. The remaining pellets were stored in separate jars, in a cupboard, at room temperature. DNA was extracted from two pellets from each treatment at monthly intervals for six months.

For each storage method, an estimation of the concentration of DNA recovered was made using spectrophotometric quantification. Mitochondrial DNA analysis was used to confirm the pellet DNA was from koala.

2.2.7. Spectrophotometric Quantification of Pellet DNA

To estimate the concentration and purity of the pellet DNA, spectrophotometric quantification was carried out using a Ultrospec 2100 spectrophotometer (Amersham Biotech Pty. Ltd). Pellet samples were diluted 1/50 and the absorbance measured at 260nm. The concentration of DNA in each sample was calculated using the formula:

$$\text{dsDNA } (\mu g/ml) = OD_{260} \times 50\mu g/ml \times \text{dilution factor}$$

where $50\mu g/ml$ corresponds to an OD_{260} of 1.

2.2.8. DNA samples used for microsatellite analysis.

DNA was extracted from tissue samples of twelve koalas from the Campbelltown population, using the technique in section 2.2.1. Pellet DNA was obtained from pellets of seven of these koalas and used for the blind trial of methods (section 2.2.16). The details of each of these koalas are located in Table 2.1.

Table 2.1 Campbelltown koalas from which tissue and pellet DNA was obtained during 2000-2002. The status indicates if the koala was living (A), dead (D), or unknown (Un) when the samples were extracted.

Assigned name	Name	Status	Pellet DNA obtained
L	Lyn	A	+
Na	Nathan	A	+
Ne	Newman	A	+
Bi	Brian	A	+
Ri	Rimas	A	-
G	Georgia	A	-
S	Shirley	A	+
H	Helen	A	-
Rho	Rhowyn	A	-
T	Taylor	A	-
Be	Breyk	A	+
Am	Amanda	A	-
Elle	Elle	A	-
Mar	Marlee	A	+
K	Kris	A	-
W	Wendy	A	-
Colo2[2]	Colo Heights 2	Un	-
F67	F67*	Un	-
F70	F70*	Un	-

* No name assigned to samples. [2] indicates a koala from the Colo Heights colony.

Table 2.2 lists the DNA samples which were extracted by B.A. Houlden in 1999, from tissue samples provided by Mr. S. Ward and Associate Professor Robert Close. Samples CM1 to CM38, Hm710, Y61/36, 95/1, and 95/2 were used by B.A. Houlden to construct the preliminary genetic database for the Campbelltown population in 1999 (Appendix 4).

Table 2.2. Koala DNA samples obtained from B.A. Houlden. The status indicates if the koala was living (A) or dead (D) or unknown (Un) when the samples were extracted.

Assigned Name	Name	Status
CM1[3]	Bluey	A
CM2[4]	D96-2	D
CM3	Roger	D
CM4[3]	D96-1	D
CM5	Harry	D
CM6	Mac	D
CM7[3]	D96-3	D
CM8	D95-2	D
CM9	Hodge	D
CM10	Gary	A
CM11	Kevin	A
CM12	Kath	A
CM14	Fran	A
CM15	Sarah	A
CM16	D97-1	D
CM17	Gaylene	A
CM18	Steve	A
CM19	D94-1	D
CM20	D95-2	D
CM22	Andrew	A
CM23	Jacob	A
CM24[4]	Scott	A

[2, 3, 4, 5] -indicates DNA samples were obtained from koalas in areas other than Campbelltown (2- Colo Heights, 3- Bargo, 4- Heathcote, 5- Unknown).

Table 2.2 (cont.). Koala DNA samples obtained from B.A. Houlden. The status indicates if the koala was living (A) or dead (D) or unknown (Un) when the samples were extracted.

Assigned Name	Name	Status
Hm710[3]	D93-1	D
Y61/36[3]	Bridgett	A
95/1[3]	D95-1	D
95/2	Casey	D
CM27	Orin	A
CM28	Leslie	A
CM30	Alan	A
CM31	Ray	A
CM33[4]	Sandy II	A
CM34[3]	Richard	A
CM35	Terry Dickey	A
CM36	Danae	A
CM37	Alby	D
CM38[4]	Heath	D
Clncy[2]	Clancy	Un
Norm[2]	Norman	Un
CB1[5]	CBTW1	Un
CB2[5]	CBTW2	Un
Sttn[5]	Sutton	Un
EO1	EO1	Un
Colo1[2]	Colo Heights 1	A

[2, 3, 4, 5] -indicates DNA samples were obtained from koalas in areas other than Campbelltown (2- Colo Heights, 3- Bargo, 4- Heathcote, 5- Unknown).

2.2.9 Amplification of microsatellites using the polymerase chain reaction

Microsatellite loci were amplified in thin walled 0.3ml omnistrips (ABgene, Pty, Ltd) using an OMN-E U well thermal cycler (Integrated Sciences). The reaction conditions were selected based on advice from Dr. M. Campbell (U.W.S), Dr. M. Eldridge (Maquarie University), Brown (2000) and Houlden *et al.* (1996a).

The primers to flank microsatellite loci were synthesised by Gensetoligos (Proligo Australia Pty Ltd) and stored at -20°C. Primers were diluted to a working concentration of 0.5- 1.0 μM. Each reaction for pellet samples, contained 1x Mg free Thermophilic buffer [Integrated Sciences, Pty, Ltd. or Astral Scientific Pty, Ltd.]; 1.5- 2.5 mM MgCl2 [Integrated Sciences, Pty, Ltd. or Astral Scientific Pty, Ltd.]; 0.5 U *Taq* polymerase in buffer IV [Integrated Sciences, Pty, Ltd.] or 0.5U BioTaq polymerase [Astral Scientific Pty, Ltd.]; 200 μM of dGTP, dCTP, dTTP, 20 μM dATP [Promega]; 0.5- 1.0 μM each desalted primer [Gensetoligos] and 0.5 μCi of [α-^{33}P]-dATP [Perkin-Elmer]. Table 2.3 lists the PCR conditions used for each primer pair.

Depending on the reaction, 1 μl of tissue DNA or 1- 2 μl of purified faecal DNA was added to each reaction tube, giving a total volume of 10μl. All faecal DNA reactions were performed in triplicate. For all faecal DNA reactions, a positive control containing koala tissue DNA was set up. Two negative controls were also set up for each locus- a non- template control to test for reagent contamination and an extraction control to test the purity of the DNA extractions. Aerosol resistant pipette tips (Molecular Bio-Products) were used in all material transfers and, to prevent evaporation, reactions were overlaid with 25μl of liquid paraffin (Fauldings).

PCR tubes were transferred to an OMN-E U well thermal cycler (Integrated Sciences). The program in section 2.2.5 was used, with the annealing time reduced to 10 seconds, to decrease non-specific annealing. For those primers which failed to give clear amplified product, the annealing temperature was increased to 57°C or a touch down program was used which involved an initial annealing temperature at 60°C with the annealing temperature decreasing by 1°C every two cycles, followed by 24 cycles using an annealing temperature of 55°C.

Table 2.3: Microsatellite loci assessed for use in tissue and faecal DNA reactions

Locus/ primer set name	Repeat motif	5' to 3' sequence	PCR Conditions used*
Phc-1[1]	$(CA)_{18}$	TGCTTGTTGACTGAATGATC	60°C and 60→55°C, $MgCl_2$/primer varied
		GAGACAGCCATCGTTTGGTT	
Phc-2[1]	$(CA)_{31}$	TGGCAAACTACTCCAGGATC	60°C and 60→55°C, $MgCl_2$/primer varied
		AGGGGGCTAATCTGGAAGAA	
Phc-4[1]	$(CA)_{29}$	AGCACAACTCTGACCATCCC	60°C and 60→55°C, $MgCl_2$/primer varied
		TCCTTAGGGAACCACTGCAG	
Phc-11[1]	$(CA)_{23}$	TACAAACTGCATCTC CCCTG	60°C and 60→55°C, $MgCl_2$/primer varied
		AGAAGGGTCTCGTGTGCTGT	
Phc-13[1]	$(CA)_{24}$	AAGCAGGATTTCATGTTCTC	60°C and 60→55°C, various [$MgCl_2$]
		GGGCAGATGAGTTTTGTTGT	
Phc-25[1]	$(CA)_{26}$	AGTTTTGTATGTGTCCGGGG	60°C and 60→55°C, $MgCl_2$/primer varied
		TCGATTGTCCTGGAGAGAAA	
K2.1[2]	$(CA)_{22}$	GCACAATAGAGCAGAGCCATAG	55°C
		GCCCATAGAAGAAATGGAAGAG	
K10.1[2]	$(CA)_{10}CG(CA)_2$	CCAATCCAAATTTAGCCAC	55°C, 1.5mM $MgCl_2$, 0.5µM primer
		CTTGGAGGTAATAGGGAAC	
Pcv 6.1[2]	$(GT)_{19}$	ACCAGGCATTTTAAACATTTGG	60→55°C
		TCAGTTTTTCCCTCTTCTTTCC	
Pcv 6.3[2]	$(TC)_{26}GTA(AC)_6$	AATCCCCGGTCCAGTAACTATC	60→55°C, 0.01% tween20, 1.5mM $MgCl_2$, 0.5µM primer
		CACTAAAGCCTTTGGGTAGGTG	
*Pcv*7.1[2]	$(TC)_{10}(AC)_8AT(AC)_6$	AATAACACCTACTTTACAAGACT	55°C, 0.01% tween 20, 1.5mM $MgCl_2$
		TAGGGAAATGAGACCCAGAAAG	
*Pcv*7.3[2]	$(GT)_7GGGG(GT)_{20}$	TTAGTCACAACACCTCCACTGC	55°C and 60→55°C
		ACCTTGAAAAATTCCCCTTTTC	
Pcv 21.3[2]	$(GT)_2(AC)_{18}$	TTGCTCCATGATAAACAACTAGT	55°C
		TGGTGAAGAAAAGGATCCATACA	
Pcv 25.2[2]	$(AC)_{15}$	GAGGCATATTTGGCTCCACCAC	55°C, 0.01% tween 20, 1.5mM $MgCl_2$
		GGGAAAAAGTTAAGTGGCTAAAT	
Pcv 32[2]	Unknown	CTCCACCCAAATATCCCTCA	55°C, 0.01% tween 20, 1.5mM $MgCl_2$
		GGCCTAAAATAGGTGTGTGTG	
Pcv 18.1[2]	$(AC)_{17}(AG)_2$	TAAAATGGTGGGGATTTAAGAAT	55°C and 60→55°C
		TCAAAGACGGGACTGGAGATTTA	
Pcv 3.2[2]	$(CA)_{28}$	ATGGCTTGAATGCTGACTTTG	55°C and 60→55°C
		CTGGCTTTTGGGCAGTAGAG	
Pcv 3.9[2]	$(GT)_{29}$	AAATACACACTGATTGGCTTGC	55°C and 60→55°C
		TGCACTTATGTTTCCACTTTTCTC	

[1] primers designed by Houlden *et al.* (1996) (sequences cited in Brown, 2000).

[2] primers designed by Cahill (2001).

* The working concentration of all primers and $MgCl_2$ was 1µM and 2.5mM respectively, unless otherwise stated. The annealing temperature used/ trialed for individual loci are listed. →denotes a touchdown PCR.

2.2.10 Preparation of polyacrylamide gel

A 21 x 40 cm Sequi-Gen® Nucleic Acid Sequencing Cell and 6% urea-polyacrylamide gel was used to separate PCR products. The apparatus and gel were prepared in accordance with manufacturer's instructions (Bio-Rad Laboratories, Pty, Ltd) and advice from Mr. F. Hinds (U.W.S).

Both glass plates were cleaned thoroughly with deionised water and dried with lint free tissue (Kim Wipes). This procedure was repeated using 70 % ethanol. The forward facing plate (attached to the upper buffer chamber) was then siliconized with Rain-x® (Unelko Corp.). After rinsing the forward facing plate with deionised water, the gel apparatus was assembled according to the Biorad Sequi-Gen Nucleic Acid Sequencing cell instruction manual.

A casting gel was prepared using Sequagel-6, a ready-to-use 6% sequencing gel solution (National Diagnostics). A 30 ml solution of Sequagel complete buffer and Sequagel-6 monomer concentrate (1:4 ratio) was mixed with 280µl freshly prepared 10% (w/v) ammonium persulfate (APS; Promega) and 240µl of Tetramethylethylene Diamine (TMED; Austral Scientific). The solution was quickly poured into the casting tray and left to polymerise for 10 min.

The running gel was prepared with a 35 ml solution of Sequagel complete buffer and Sequagel-6 monomer concentrate (1:4 ratio) mixed with 320µl freshly prepared 10% (w/v) APS. The running gel was cast according to the Biorad Sequi-Gen Nucleic acid Sequencing cell instruction manual. Once cast, the straight edge of the well comb was inserted across the top of the gel and clamped into place. The gel was then left to polymerise for 1.5 –2 hours.

2.2.11 Preparation of DNA marker for polyacrylamide gels

M13mp18 DNA markers, G and A, were prepared separately using a T7 Sequenase version 2.0 DNA sequencing kit (Amersham) in accordance with manufacturer's instructions. An annealing reaction was prepared consisting of 2 µl T7 Sequenase reaction buffer, 1 µl sequenase primer (-40 M13, 0.5 pmol μl^{-1}), 5µl M13mp18 single stranded DNA (0.2 $\mu g\ \mu l^{-1}$) and sterile deionised water (Milli-Q) to a final volume of

10µl. The annealing reaction between the M13mp18 DNA and the sequenase primer was carried out by heating for 2 min at 65 °C and then cooling slowly to 30 °C. The DNA was labeled by incubating the annealing solution at room temperature for 5 min, with 1 µl dithiothreitol (DDT; 0.1 M), 2µl labeling mix (25x), 0.5 µl [α-^{33}P]-dATP and 2 µl T7 sequenase polymerase (12.5x). A terminating reaction was performed by adding 3.5 µl labeled reaction solution to 2.5 µl terminating solution and incubating for 5 min at 37° C. This reaction was stopped by adding 4 µl STOP solution. Markers were denatured at 80° C for 5 min prior to loading onto a sequencing gel.

2.2.12 Polyacrylamide gel electrophoresis of PCR products

The Sequi-Gen Nucleic Acid Sequencing cell was assembled for operation according to the manufacturer's instructions. A (1x) 0.089 M Tris, 0.089 M Borate, 0.002 M EDTA (TBE) buffer was prepared from a 10x TBE concentrate (Amresco®) and 500 ml added to the upper and lower buffer chambers. The cell was pre-run at 60 W for 30 min or until the temperature reached 45-50 °C. A 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 90% (v/v) formamide loading buffer (4µl) was added to all samples (10µl) containing PCR products and the DNA denatured at 80°C for 5 min prior to loading the samples on to the gel. The products were separated by electrophoresis at 60 W for 1-2 hours. The size of the fragments, amplified in PCR, were obtained by comparison with ^{33}P labelled M13mp18 DNA markers loaded onto the gel (G and A, 3µl each). The running time varied depending on the expected size of the PCR products and the migration of bromophenol blue (~26bp) and xylene cyanol (~110 bp) dyes.

2.2.13 Gel drying and autoradiography

On completion of electrophoresis, the glass plates were separated and the gel carefully transferred onto Whatman 3M filter paper. The gel was covered in Glad Wrap and placed face up in a BioRad Model 538 gel dryer. Drying was carried out for 45-60 min, on a sequencing cycle at 80 °C, with a BioRad HydroTech vacuum pump.

The dry gels were then transferred to a Kodak intensifying cassette for autoradiography with Hyperfilm MP (Amersham). The exposure was carried out at -70°C for 48-72 hours. The exposure time was varied depending on the age of the ^{33}P.

2.2.14 Film Development

Films were developed under a Kodak No.2 light filter, in Kodak GBX developer/ replenisher for 5 min and rinsed for 30s under running water. The film was then placed in Kodak GBX fixer/ replenisher for 10 min and rinsed in water for 2 min before air-drying.

2.2.15 In-house blind trial

To determine whether the above methods could be used in the field to identify individual koalas, an in-house blind trial was conducted utilising faecal DNA extracts previously obtained from seven known individuals from the Campbelltown area (see Table 2.1). Three colleagues were asked to each choose three faecal DNA extracts and to aliquot 70 µl of each extract into separate 1.5 ml microcentrifuge tubes labeled with their initial and a number from one to three. The identity of each sample was withheld and microsatellite analysis was carried out on each unknown sample using the methods described in sections 2.2.9- 2.2.14, and Cahill's (2002) primers (Table 2.3).

Allele sizes obtained for each unknown sample were compared to existing genotypic data for each locus and a tentative identity assigned to each sample. The true identity of the unknown samples was revealed by each colleague who aliquoted out the faecal sample for analysis.

2.2.16 Analysis of genotypic data for tissue samples

Allele sizes were estimated by comparison to the A and G M13mp18 ssDNA markers. Allele sizes for each locus were put into Excel and a measurement of genetic diversity at each locus was made using the software package GenAlEx : Genetic Analysis in Excel (Peakall and Smouse, 2001). The observed heterozygosity (H_O), the level of heterozygosity expected from Hardy-Weinberg assumptions (H_E) and the inbreeding coefficients (F- statistics) were determined for each locus. The rate of migration for the Campbelltown koalas between areas of Southern Sydney was also determined using the following equation:

$$Nm = (1 - F_{ST})/ 4 F_{ST}$$

Where N is the effective population size and m is the migration rate (Wright, 1969).

Genotypic data was examined to test conformity to the Hardy Weinberg Equilibrium model for each population of koalas using the software package Genepop version 3.3 (Raymond and Roussett, 1995). The test for Hardy Weinberg proportions for more than five alleles is a Markov chain method and this was used to obtain unbiased estimates of exact probability. A significant deviation from Hardy Weinberg expectations was found when $P < 0.05$ and no significant departure was detected when $P \geq 0.05$.

The ability of Houlden *et al*'s (1996) and Cahill's (2001) microsatellite markers (Table 2.3) to distinguish between individual koalas in a population, i.e. probability of identity, was estimated using allele frequencies for each locus. The freeware program Identity 1.0 (Wagner and Sefc, 1999) was used to calculate the probability of a genetic match among random pairs of unrelated individuals (PI). The probability of distinguishing between full siblings (PI_{sibs}) was also calculated using the following formula (Waits *et al*, 2001):

$$PI_{sibs} = 0.25 + (0.5 \sum p_i^2) + [0.5 (\sum p_i^2)^2] - (0.25 \sum p_i^4)$$

Where p_i is the frequency of the ith allele.

2.2.17 Animal ethics, radiation safety and project approval

The collection of tissue samples and the use of ^{33}P ionizing radiation were approved by the Animal Care and Ethics Committee and the Biosafety and Radiation Safety Committee of the University of Western Sydney.

3.0 Results

3.1 DNA extractions from tissue

Koala genomic DNA and water rat DNA were extracted from tissue samples using the protocol described in section 2.2.1 Electrophoresis of the DNA on an agarose gel showed most of the DNA as a high, molecular weight band near the top of the gel (Figure 3.1). The method produced mostly intact and clean DNA. The amount of DNA present was estimated by comparing the band intensity to that of the SPP-1 size marker (Appendix 1). Between 80ng to 2640ng of koala DNA in a final volume of 200µl, was recovered from each extraction. Approximately 21 µg of water rat DNA was recovered.

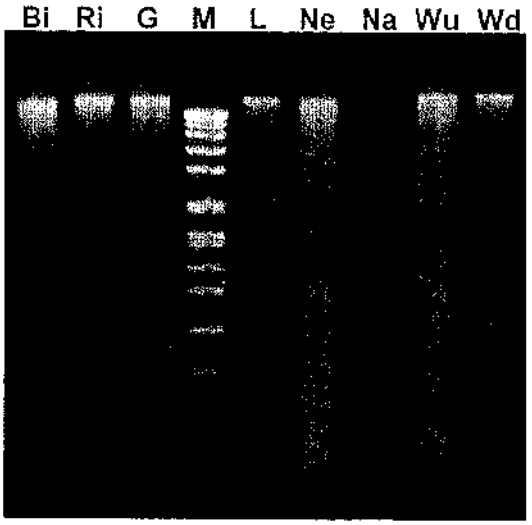


Figure 3.1: Genomic DNA extractions for six Campbelltown koalas and a water rat, shown on a 2% agarose gel. The DNA was extracted from ear punches of these koalas: Brian (Bi), Rimas (Ri), Georgia (G), Lyn (L), Newman (Ne) and Nathan (Na). The water rat DNA (W) was obtained from a liver sample. An undiluted (Wu) and 1/10 dilution (Wd) sample are shown. M represents the SPP-1 marker.

PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.

3.2 DNA extractions from koala faecal pellets

3.2.1 Spiked pellet experiment

To ensure the pellet DNA extraction procedure would work, water rat DNA was spiked onto a koala faecal pellet, extracted using the method in section 2.2.2 and used for microsatellite analysis (section 2.2.4). Figure 3.2 shows a comparison of the results obtained from a tissue sample and the DNA recovered from the surface of a koala faecal pellet. The tissue (T) produces bands of a greater intensity than the spiked DNA replicates, indicating that only a small amount of the original 10ng of spiked DNA was recovered from the surface of the pellet. The tissue bands clearly match the bands in the pellet samples (Figure 3.2), proving that the surface wash method for extracting DNA from faecal pellets can be effective.

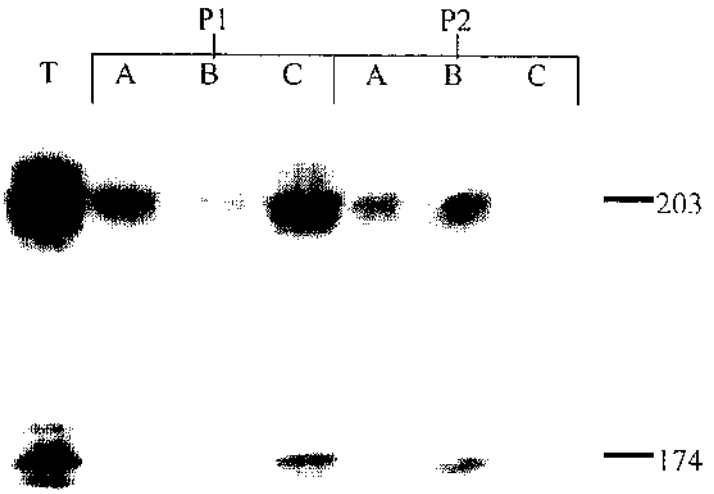


Figure 3.2: Autoradiograph of microsatellite locus W2A1 showing amplified water rat tissue DNA (T) and water rat DNA recovered from the surface of two spiked koala pellets (P1 and P2). Pellet samples were amplified in triplicate. Sample C from pellet 2 is visible but very faint and matches all other replicates. The numbers refer to allele sizes in base pairs. The negative control (not shown) was not contaminated.

3.2.2 Faecal DNA extractions from koala pellets

The koala pellet easily absorbed moisture and soon became brittle. Therefore the surface wash procedure needed to be completed in a short period of time, ≤ 1 minute for both washes. Approximately 200 µl of the first wash (400 µl) was absorbed by the pellet allowing the remaining solution to be used for the DNA extractions. None of the second wash buffer was absorbed by the pellets. In cases where the whole of the first application of PBS was absorbed a further two washes were required using 200 µl PBS each time. Any pellet damaged prior to or during the first surface wash was not used.

3.2.2.1. Optimal incubation time for buffers ASL and AE

Initial attempts made during this study to recover koala DNA from pellets, using the manufacturer's protocol, proved fruitless. Insufficient DNA was recovered to visualise on an agarose gel and mitochondrial DNA analysis of these first attempts failed. Initial faecal DNA extractions were carried out using an incubation time of five minutes for buffer ASL. Varying this time between 5-60 min increased the yield of DNA recovered from the pellets. Table 3.1 shows the optimal time the surface washes should be incubated with the Qiagen lysis buffer ASL was between 45- 60 minutes.

The incubation time for buffer AE, the elution buffer which recovers DNA from the spin column, was also increased to five minutes, to improve the elution efficiency

The changes to the initial and final buffer incubation times recovers sufficient DNA from the pellet to be visualised on an agarose gel. Mitochondrial DNA PCR analysis of the extracts confirmed these results. Relevant electrophoresis results are given in the following sections.

Table 3.1: Recovery of DNA from the surface of koala faecal pellets after varying the time of incubation with buffer ASL at 35°C. * poor recovery, ** good recovery, *** excellent recovery, – DNA not detected.

Time (min)	Visible on 2%Agarose gel	mtDNA amplified
5	-	-
10	-	-
15	-	*
30	-	*
45	*	**
60	***	***

3.2.2.2 Faecal DNA

Total faecal DNA appears as high molecular weight bands after electrophoresis (Figure 3.3). In all cases (~70 extractions) using the optimal ASL incubation time, the pellet extraction methods appear to produce clean DNA extracts, which can be visualised on agarose gels. The total amount of DNA extracted from the pellets ranges between less than 30ng to 140ng in a final volume of 160µl. The amount of DNA recovered from the pellets was proportional to the colour of the surface washes. More DNA was recovered when the surface washes were a mid to dark brown colour.

The method for extracting DNA from koala pellets refined in this study has been trialled by inexperienced laboratory workers to test its ease of use. These trials produced results such as those in Figure 3.3a and 3.3b, confirming that the adjusted method of extracting DNA from faecal pellets, described in section 2.2 is reliable and reproducible.

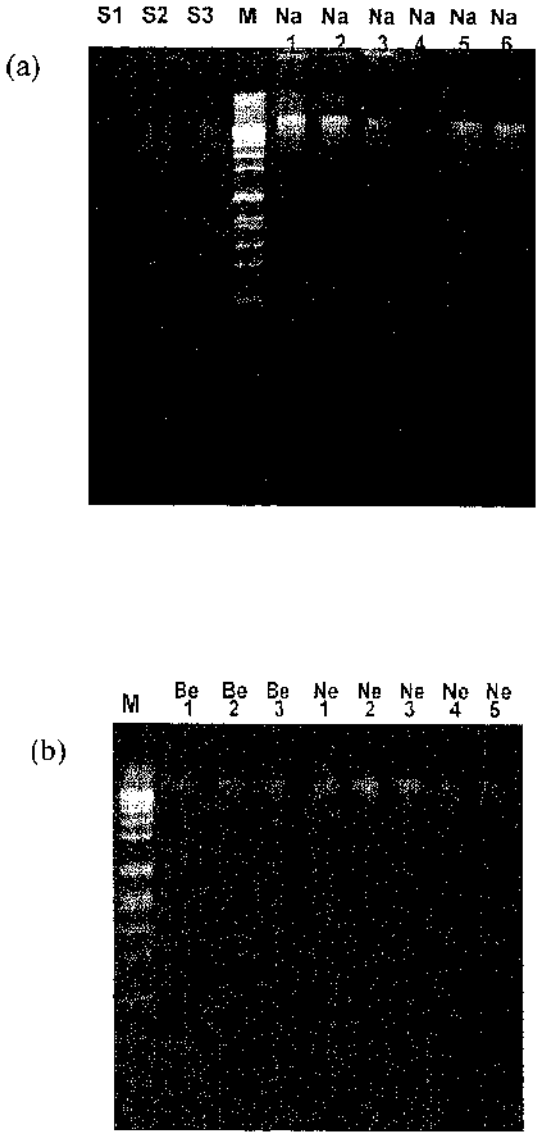


Figure 3.3 Examples of DNA extractions obtained from surface washes of faecal pellets. (a) Pellets obtained from Nathan (Na) and Shirley (S). (b) Pellets obtained from Breyk (Be) and Newman (Ne). Pellet DNA samples were run in conjunction with an SPP-1 marker (M).

3.2.3 Mitochondrial analysis of faecal DNA samples.

The 5' end of the mtDNA control region (D-loop) was successfully amplified from koala genomic and faecal DNA using primers 15996L and 16502H. Electrophoresis of the mtDNA products on an agarose gel showed bands for both tissue DNA and faecal DNA travelling approximately the same distance (Figure 3.4). These bands were all found to be approximately 832bp in length, close to the ~850bp found by Houlden *et al.* (1999), suggesting that the faecal DNA was koala. All extracts of faecal DNA used in the present study (~70) amplified the 832bp mtDNA fragment.

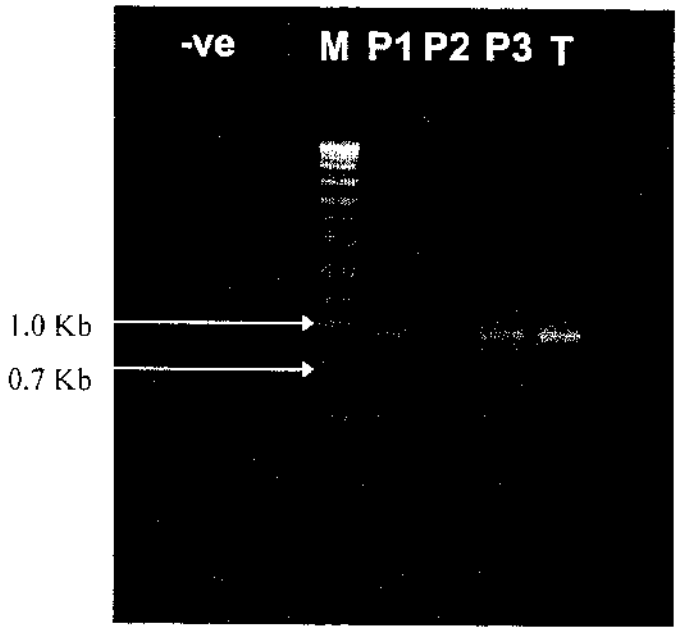


Figure 3.4: Example of PCR amplification of mtDNA extracted from koala faecal pellets. A tissue sample (T) run in conjunction with the three faecal samples (P1, P2 and P3) produced a band of a similar size to all faecal bands. A size comparison to the SPP-1 marker (M) shows all bands to be ~ 832bp. The negative control shows no contaminants present in the reactions.

3.2.4. Faecal DNA preservation studies

3.2.4.1 Conservation of DNA on wet pellets

Figures 3.5 shows the mtDNA PCR results of faecal DNA extractions performed on five fresh pellets and sections of various types of paper, which had been wrapped around the pellets prior to drying. An amplifiable product was obtained for all DNA extractions performed on the various types of paper (paper towel, tissue, filter paper from a microscope slide box and Whatman 1mm filter paper). The intensity of these products was indistinguishable from the pellet extracts, indicating that a significant amount of cells on the surface of the pellets can be shed and dried onto paper. Therefore, wet pellets in the field should be individually wrapped in a small section of paper prior to collection and drying. A DNA extraction should be carried out on the paper in conjunction with the DNA extraction from the pellet and the paper and pellet extracts pooled to ensure the maximum amount of DNA is recovered from each sample.

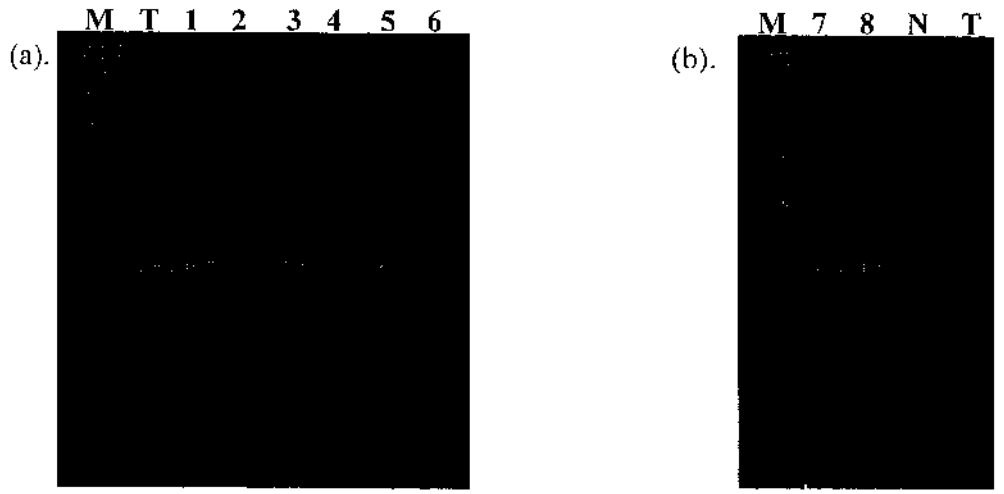


Figure 3.5 MtDNA PCR results of faecal DNA extractions performed on five fresh pellets and sections of various types of paper. (a) Pellet and paper samples respectively from paper towel (1 & 2), tissue paper (3 & 4), and filter paper (5 & 6) from a microscope slide box. (b) Paper and pellet sample from Whatman 1mm filter paper (7 & 8), negative control (N). All PCR reactions were run in conjunction with a DNA sample from tissue (T) and the SPP-1 size marker (M).

3.2.4.2 Optimal pellet storage conditions

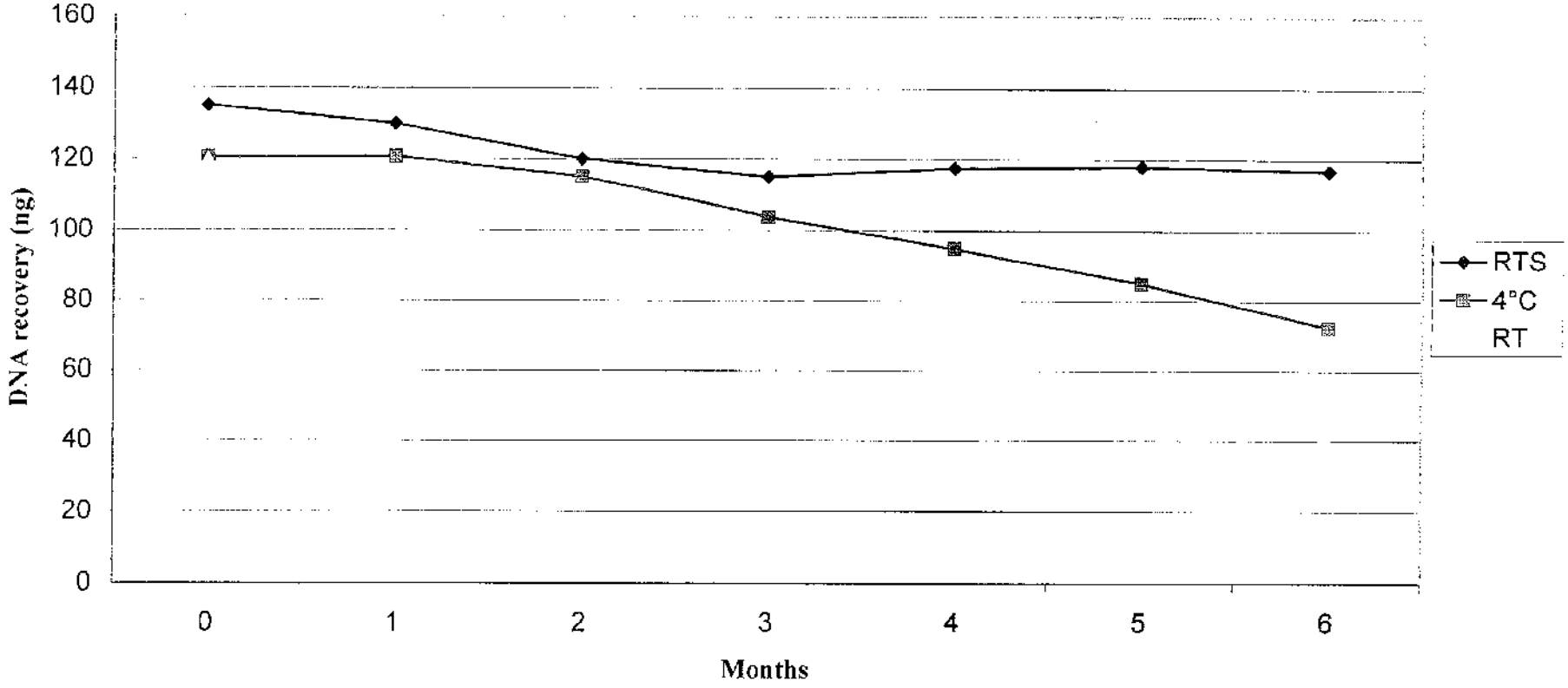
The optimal method of DNA preservation was to spray the pellets with 70% ethanol and store them at room temperature (RTS), which conserved the DNA for up to six months without substantial loss or degradation of the DNA. A 13% reduction in the amount of DNA recovered after six months was observed, using this method of preservation.

The second method of DNA preservation, storage at 4°C, also fared well for 2-3 months of storage. However, after this time a rapid decrease in the quantity of DNA recovered from the pellets was observed. The total loss of DNA after six months was approximately 40%.

In contrast, the amount of DNA recovered from pellets stored at room temperature (RT) fell sharply after two months, with only 25% of DNA able to be recovered after six months. The DNA preservation results are shown in Figure 3.6.

DNA was also preserved when pellets were stored at -70°C for six months. The amount recovered from the frozen pellets could not be measured using spectrophotometric analysis, but was shown to be present using mitochondrial DNA analysis.

Figure 3.6: Faecal DNA recovery over six months using various preservation methods



3.3 Amplification of microsatellites.

3.3.1 Optimisation of PCR conditions for Houlden *et al.*'s (1996a) six primer sets

Repeated problems with the amplification and identification of alleles using the six primers of Houlden *et al* (1996a) (see Table 2.3) were experienced. The turnaround time for the PCR and autoradiographs resulted in the PCR optimisation for these primers taking up a significant portion of the research year.

A number of variations to the PCR conditions were trialled. These included:

- Varying the pH of the reaction buffer between 8.3- 9.2;
- Varying the $MgCl_2$ concentration between 1.0-2.5 mM;
- Altering the primer annealing temperature between 50-60 °C, using a gradient PCR;
- Lowering the annealing time from 30 s to 10s;
- Varying the concentration of primers between 0.2- 1μM.
- Altering the recommended dilution of the DNA samples used for optimisation from 1in 100 to 1 in 500 and 1 in 1000.

Adjusting the concentrations of PCR reagents and cycling parameters had little or no effect. Even the use of a high performance *Taq* (BioTaq) failed to improve the results. There was no evidence that the DNA contaminated in the negative controls.

Figure 3.7 shows the typical results obtained for loci *Phc*-1, 2, 4, 11, 13 and 25, using DNA samples CM1, CM2 and CM3 (Table 2.2). The negative control (N) is indicated and the M13mp18 marker (A and G), used for sizing the alleles, is also shown.

Phc-13 was the only locus which amplified consistently and clearly. Non- specific bands were present in some reactions for this locus, but these bands did not interfere with the identification and sizing of the alleles. The allele sizes obtained for this locus, 111 and 117bp, match the results obtained by B. A. Houlden for the preliminary genetic database (Appendix 4).

The sizes of the alleles at *Phc*-1 (Figure 3.7) differ from the expected results (Appendix 4) by ~19bp as the forward primer used for this locus contained an M13 tail. Other attempts to amplify this locus, using primers without this tail, failed. The sequence of the primers supplied by the manufacturer was examined and the forward primer contained an error. This error was not present in the sequence submitted to the company.

Phc- 2, 4, 11 and 25 contained non-specific bands or smeared DNA patterns for most reactions, making it difficult to accurately identify the position of individual alleles. The optimisation strategies listed above decreased the level of non specific banding occurring for loci *Phc*- 2, 4 and 11, allowing the alleles to be identified and sized, but failed to reduce the appearance of non-specific bands and smearing for locus *Phc*- 25. Subsequent reactions again produced non-specific band which interfered with allele identification and sizing. The allele sizes match the expected results for locus *Phc*-4 and differ from the expected results by 2-3bp for loci *Phc*-2 and 11, most likely due to a difference in the marker used for sizing alleles or slippage of the *Taq* during PCR.

The failure to produce any meaningful results using five of the six loci, *Phc*-1, 2, 4, 11, 13 and 25 (Houlden *et al.* 1996a), prevented these primers from being used for faecal DNA analysis.

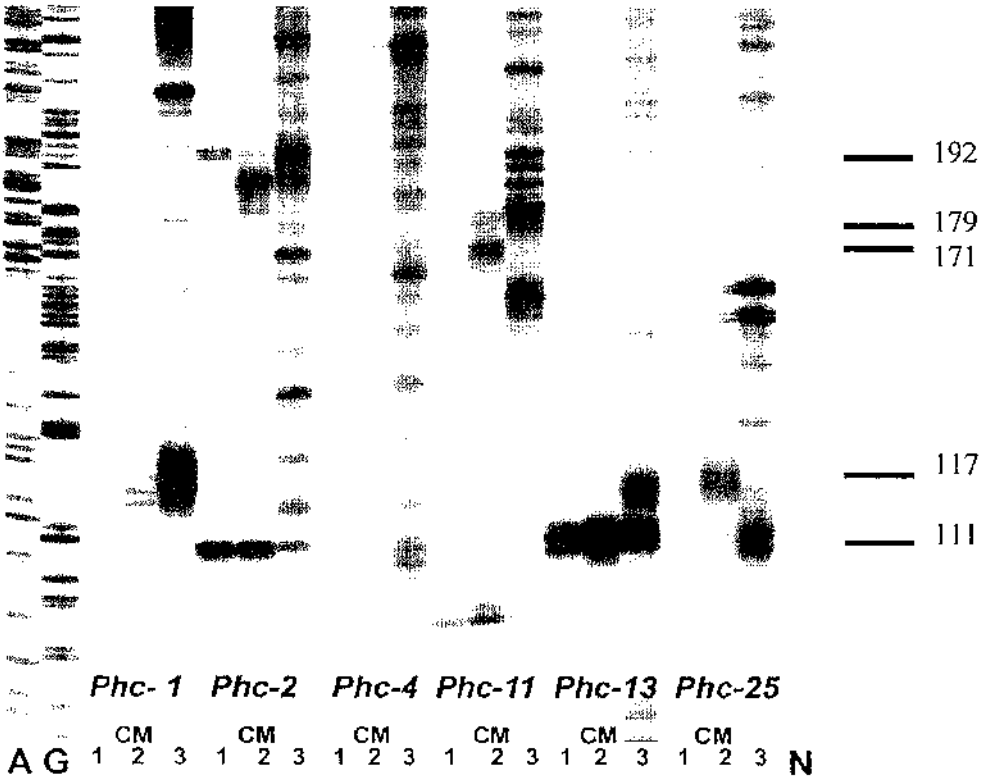


Figure 3.7 Autoradiograph of microsatellites amplified using primers *Phc*-1, 2, 4, 11, 13 and 25 and DNA samples CM1, CM2 and CM3. Non-specific bands and smeared DNA surround each locus. Allele sizes are in base pairs. The negative control is free from contamination.

3.3.2. Optimisation of PCR conditions for Cahill's (2001) primers

Twelve microsatellite primers sets designed by Cahill (2001) (Table 2.3) became available during the current project and were immediately trialled. Eight of these primer sets, K 2.1, K10.1, *Pcv* 6.1, 6.3, 7.1, 21.3, 25.2 and 32, required little optimization of PCR conditions. With the exception of K10.1, *Pcv* 6.1 and 6.3, these primers amplified microsatellites easily during the first trial runs and only required the addition of 0.01%Tween 20 and/or a reduction in the concentration of $MgCl_2$ for the alleles to be clearly identified. Allele size varied at each loci by 2bp. Stutter bands were amplified at all loci. Only the darkest bands were scored as the alleles at each locus.

A touchdown PCR was used to encourage primer annealing and amplification for the reluctant primers and successfully amplified *Pcv* 6.1 and 6.3. Unfortunately, the intensity of the amplified bands for locus *Pcv* 6.1 remained faint despite increasing the exposure time, but bands could clearly be seen with the aid of a light box. A single intense band at 222bp appeared in some samples at locus *Pcv* 6.1 (see figure 3.10, sample T and CM3). Subsequent reactions using the samples amplifying this band revealed that this band was an allele for this locus, as very faint stutter bands surrounding the intense band were observed.

A non specific band of ~133bp appeared in most autoradiographs for locus K10.1. Initially this band prevented the accurate identification of the actual alleles. The intensity of this band was decreased in heterozygous individuals, or removed from homozygous individuals when the amount of DNA and primer used in the reaction was decreased.

A touchdown PCR also prompted *Pcv* 7.3 and 3.9 to partially amplify microsatellites from some DNA samples. However, the inconsistent results obtained with these two primers prevented them from being used within this study and further optimisation is required for these primers to ensure they will be of use in future studies.

Loci, *Pcv* 18.1 and 3.2, consistently failed to amplify. Touchdown PCRs did not amplify these loci and increasing the amount of primer had no effect. Other optimisation strategies such as adjusting the pH of the buffer and the amount of *Taq* were also unsuccessful.

Figures 3.8 to 3.15 show the typical results obtained for loci K 2.1, K10.1, *Pcv* 6.1, 6.3, 7.1, 21.3, 25.2 and 32, using DNA samples listed in Tables 2.1 and 2.2. The M13mp18 marker (A and G), used for sizing the alleles, is shown. Lanes containing a negative control are not shown as no contamination was present for these reactions.

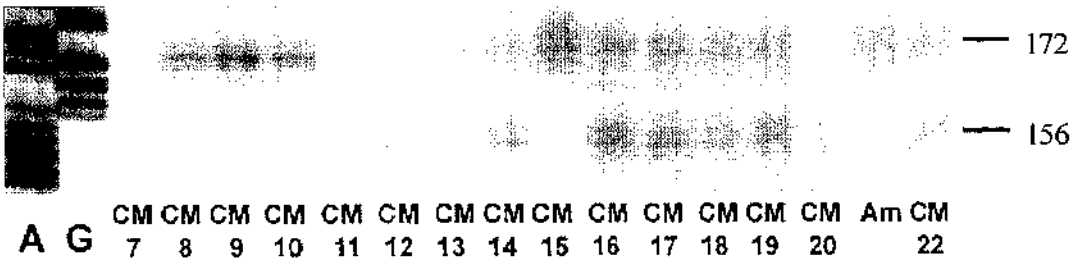


Figure 3.8 Example of an autoradiograph of microsatellites amplified using primer set K2.1. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs. Non specific bands were present in some samples, but were not located near the alleles.

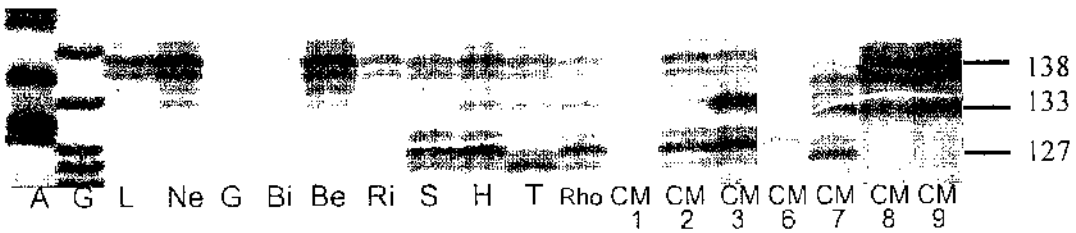


Figure 3.9 Example of an autoradiograph of microsatellites amplified using primer set K10.1. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs. The negative control is not shown. Note the intensity of the false allele at 133bp in some samples; this band was removed when the reactions were repeated using a lower concentration of primer.

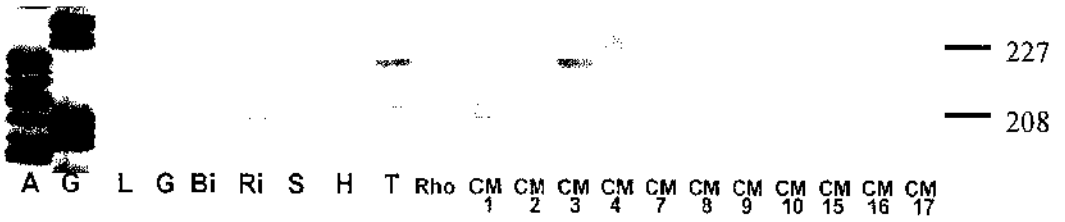


Figure 3.10 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 6.1. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs. Note the poor intensity of many of the samples. These can be clearly seen with a light box.

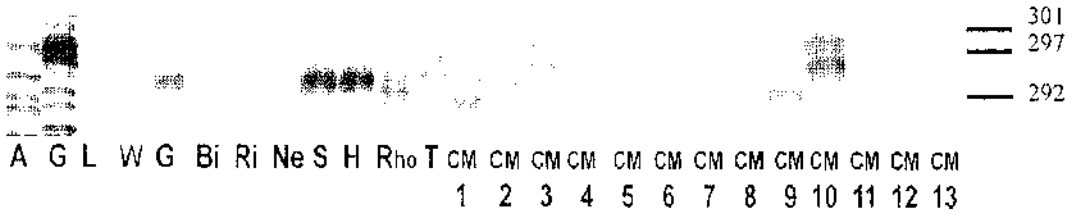


Figure 3.11 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 6.3. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs. Non specific bands were evident in a few reactions at ~ 90bp.

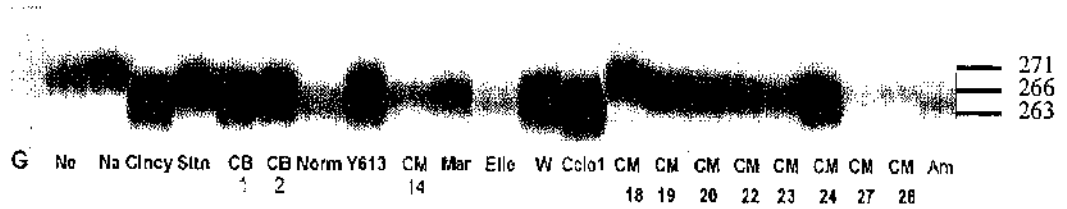


Figure 3.12 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 7.1. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, G represents the M13mp18 DNA marker. Allele sizes are in base pairs. Note the high intensity bands produced in most reactions. Alleles can be visualised using a light box.

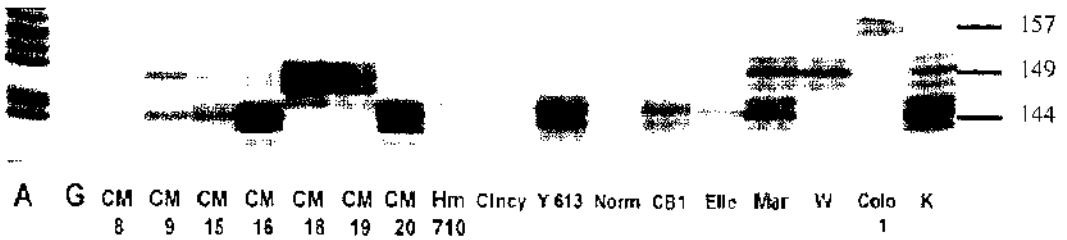


Figure 3.13 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 21.3. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2. A and G =M13mp18 DNA marker. Allele sizes are in base pairs. Single non- specific bands were amplified in most samples at ~100bp and at >250bp, but did not interfere with the identification or sizing of the alleles. A contaminant was present for some samples and corresponded to some of the non-specific bands.

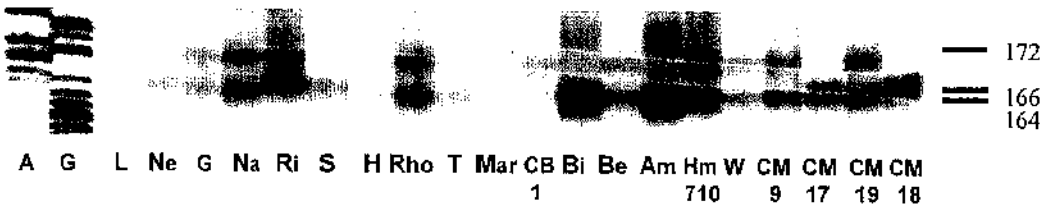


Figure 3.14 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 25.2. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs.

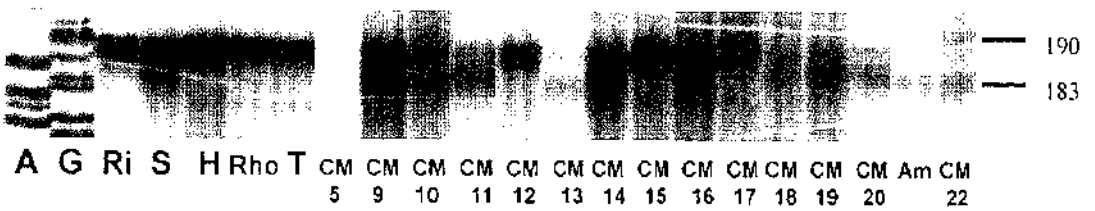


Figure 3.15 Example of an autoradiograph of microsatellites amplified using primer set *Pcv* 32. The samples indicated on the figure correspond to individuals listed in Table 2.1 and 2.2, A and G =M13mp18 DNA marker. Allele sizes are in base pairs. Single non- specific bands were amplified in most samples at ~100bp and at >250bp, and did not affect the identification and sizing of the alleles.

3.3.3 Microsatellite genotyping of koala tissue samples using optimized primer sets

Microsatellite genotyping was performed on a total of 63 individuals, using primer sets K 2.1, K10.1, *Pcv* 6.1, 6.3, 7.1, 21.3, 25.2 and 32. Forty-six of these individuals were sampled in the Campbelltown area, six in the Colo Heights area, seven in the Bargo area and four in the Heathcote area (see Figure 1.1 for these locations) The area in which 'Sutton', 'CBTW1' and 'CBTW2' were sampled are unknown. The level of genetic variation detected was high enough to generate a unique genetic profile for each of these koalas (Table 3.2).

Some tissue samples failed to amplify at selected loci. These samples were repeated using an increased amount of DNA. This solution worked for most loci, however some samples, such as CM5, did not produce a result even after increasing the amount of DNA in the reaction. Failed PCR reactions are listed as '0' in Table 3.2.

The allele sizes for each locus are summarised in Table 3.3.

Table 3.2 Genotype data for eight microsatellite loci for 63 koalas

		Allele size in base pairs															
Assigned Name	Area of Origin*	K2.1		K 10.1		*Pcv* 25.2		*Pcv* 6.1		*Pcv* 6.3		*Pcv* 7.1		Pcv 21.3		Pcv 32	
CM1	3	158	158	127	127	166	166	0	0	285	293	262	262	144	144	193	193
CM2	4	158	158	127	139	166	172	208	208	289	289	260	262	150	150	195	195
CM3	1	158	168	127	139	164	164	208	222	293	301	268	268	150	162	183	189
CM4	3	158	158	127	139	164	164	222	222	293	293	266	272	152	152	183	189
CM5	1	0	0	0	0	164	164	206	206	0	0	262	272	0	0	0	0
CM6	1	168	172	129	129	164	172	206	208	283	281	260	268	144	144	181	181
CM7	3	158	172	135	127	164	164	208	228	285	293	268	268	144	144	181	189
CM8	1	172	172	139	139	164	164	208	228	295	301	268	268	144	150	189	189
CM9	1	172	172	139	139	164	172	208	228	293	301	262	268	144	150	183	189
CM10	1	172	172	139	139	164	164	208	228	293	293	266	272	144	144	183	189
CM11	1	158	172	139	139	164	172	208	228	293	293	268	268	144	150	183	189
CM12	1	158	158	127	139	164	172	208	208	293	293	268	268	144	150	189	189
CM14	1	158	172	139	139	164	172	208	208	285	301	268	268	144	150	183	189
CM15	1	174	174	139	139	166	172	228	228	285	289	268	268	144	144	190	189
CM16	1	158	174	127	139	164	164	208	228	295	301	268	268	150	150	181	189
CM17	1	158	172	127	139	164	166	208	228	289	295	268	268	150	150	189	189
CM18	1	158	172	139	139	166	166	208	228	295	301	272	272	144	144	183	189
CM19	1	158	172	139	139	164	172	208	208	295	301	266	268	142	144	183	189
CM20	1	158	172	139	139	164	172	208	208	295	295	262	268	144	144	183	189
CM22	1	158	172	139	139	166	172	208	208	295	295	266	266	144	150	181	181
CM23	1	172	172	139	139	164	172	0	0	0	0	262	262	150	150	183	183

* Area the koala was found/ sampled: 1- Campbelltown, 2- Colo Heights, 3- Bargo, 4- Heathcote, 5- Unknown.
0- Indicates a PCR reaction which repeatedly failed.

Table 3.2 (cont.) Genotype data for eight microsatellite loci for 63 koalas

		Allele size in base pairs							
Assigned Name	Area of Origin*	K2.1	K 10.1	*Pcv* 25.2	*Pcv* 6.1	*Pcv* 6.3	*Pcv* 7.1	Pcv 21.3	Pcv 32
CM24	4	174 174	139 139	164 164	228 228	293 295	260 268	144 150	183 189
Hm710	3	158 168	127 127	164 172	0 0	293 293	262 268	146 152	175 183
Y61/36	3	156 168	139 139	166 166	208 208	285 293	262 268	146 146	175 183
95/1	3	168 168	127 127	168 168	206 206	289 289	260 268	156 156	181 181
95/2	1	158 158	139 139	172 172	208 230	289 295	262 268	150 150	189 189
CM27	1	172 172	139 139	166 172	0 0	0 0	262 262	144 144	189 189
CM28	1	154 174	127 127	166 166	0 0	295 295	266 266	0 0	189 189
CM30	1	174 174	139 139	164 164	0 0	295 295	268 280	150 150	189 189
CM31	1	158 172	139 139	164 166	0 0	295 295	262 262	0 0	183 189
CM33	4	158 158	127 127	166 166	208 208	295 295	260 262	144 150	175 189
CM34	3	158 168	127 139	164 164	208 208	289 289	260 262	144 154	183 189
CM35	1	158 158	127 139	164 172	0 0	295 295	262 262	144 144	189 189
CM36	1	158 158	139 139	166 166	0 0	0 0	0 0	144 150	181 181
CM37	1	172 172	139 139	164 166	264 264	289 301	268 268	144 150	189 189
CM38	4	158 172	127 139	164 166	216 264	289 295	252 262	144 144	189 189
S	1	158 172	127 139	164 172	206 230	295 295	268 272	144 150	183 189
L	1	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
G	1	158 172	139 139	164 172	208 232	295 295	266 266	144 150	183 189
Bi	1	172 172	139 139	164 172	206 228	293 293	276 272	144 144	183 193
Ri	1	158 174	139 139	166 172	208 232	293 293	262 262	144 150	189 189

* Area the koala was found/ sampled: 1- Campbelltown, 2- Colo Heights, 3- Bargo, 4- Heathcote, 5- Unknown.
0- Indicates a PCR reaction which repeatedly failed.

Table 3.2 (cont.) Genotype data for eight microsatellite loci for 63 koalas

Assigned Name	Area of Origin*	Allele size in base pairs													
		K2.1		K 10.1		*Pcv* 25.2		*Pcv* 6.1		*Pcv* 6.3		*Pcv* 7.1		Pcv 21.3		Pcv 32	
Be	1	158	174	139	139	164	172	206	228	295	295	272	272	144	144	189	189
H	1	168	172	127	139	164	172	228	232	295	295	266	276	144	150	183	189
Rho	1	172	172	127	139	164	172	208	222	293	295	266	272	144	150	189	189
T	1	172	172	139	139	164	164	208	222	295	295	266	272	144	150	183	189
Na	1	158	168	127	139	164	172	208	228	297	297	272	272	144	150	181	189
Ne	1	172	172	139	139	164	166	208	208	297	297	272	272	150	150	183	189
Am	1	172	172	139	139	164	172	208	230	295	295	262	272	144	150	181	181
Clncy	2	168	168	139	139	166	172	208	208	292	301	260	266	146	150	181	181
Norm	2	158	168	129	129	172	172	208	208	285	285	260	266	144	150	189	189
CB1	5	158	158	139	139	172	172	208	228	289	295	260	268	146	146	189	189
CB2	5	168	168	139	139	164	164	204	204	289	289	262	268	0	0	181	181
Sttn	5	158	168	139	139	166	172	208	208	285	301	268	268	0	0	185	185
Elle	1	0	0	139	139	166	166	0	0	295	295	266	272	146	146	193	193
Mar	1	158	158	139	139	172	172	208	208	285	293	268	268	150	150	181	189
W	1	174	174	139	139	164	172	0	0	295	295	262	262	144	144	189	193
Colo2	2	154	158	125	139	166	170	208	208	311	311	260	262	144	144	193	193
K	1	158	174	127	127	166	166	208	232	295	295	266	272	142	142	189	189
F67	1	0	0	127	139	166	166	232	232	295	295	266	272	146	146	189	189
F70	1	0	0	139	139	166	166	212	212	289	311	262	268	146	154	175	181
EO1	5	0	0	135	135	0	0	0	0	0	0	0	0	0	0	189	189
Colo1	2	168	168	125	135	166	172	0	0	311	311	262	268	144	144	183	185

* Area the koala was found/ sampled: 1- Campbelltown, 2- Colo Heights, 3- Bargo, 4- Heathcote, 5- Unknown.
0- Indicates a PCR reaction which repeatedly failed.

Table 3.3 Summary of allele sizes for eight microsatellite loci.

Locus	No. alleles	Allele size in base pairs per locus									
K2.1	6	154	156*	158	168	172	174				
K10.1	5	125*	127	129	135	139					
Pcv 25.2	5	164	166	168*	170*	172					
Pcv 6.1	10	204	206	208	212	216*	222	228	230	232	264
Pcv 6.3	9	281	283	285	289	293	295	297	301	311	
Pcv 7.1	8	252*	260	262	266	268	272	276	280		
Pcv 21.3	8	142	144	146	150	152*	154	156*	162		
Pcv 32	7	175	181	183	185*	189	193	195*			

* Allele not detected in Campbelltown population.

The alleles possessed by individuals CBTW1, CBTW2 and Sutton for each locus allowed them to be assigned to one of the four locations sampled in this study. CBTW1 and CBTW2 were assigned to the Campbelltown population and Sutton was tentatively assigned to Colo Heights.

The two pairs of individuals, CM3 and CM16 and CM11 and CM2, which showed identical profiles using Houlden *et al's* (1996) six primer sets, were proven to be individuals after analysis with Cahill's (2001) eight primer sets. CM3 and CM16 differ by one allele at loci K2.1, *Pcv*- 6.1, 6.3, 21.3 and 32, whilst CM11 and CM20 differ by one allele at loci *Pcv*- 6.1, 7.1 and 21.3 and both alleles at locus *Pcv*- 6.3. Table 3.4 shows the comparison between these samples for all loci.

The probability of obtaining two individuals in the Campbelltown population with identical genotypes (PI) was estimated for Cahill's (2001) eight loci using the allele frequencies for the data in Table 3.2 to be 3.17×10^{-5} (~1 in 31,550) for unrelated individuals and 8.08×10^{-4} (~1 in 1240) for full siblings. No genetic matches were obtained for these eight loci among all 63 koalas. In contrast, the PI estimated using allele frequencies from Houlden's preliminary database (Appendix 4) was 1.41 x 10-3 (~1 in 700) for unrelated individuals and 1.57 x 10-2 (~1 in 64) for full siblings and two pairs of genetic matches were found.

The power of Cahill's (2001) eight primers to distinguish between individuals with closely shared genotypes, establishes the prospect of successfully using faecal DNA for this purpose.

Table 3.4: Comparison of genotypes for CM3 and 16, and CM11 and 20 for all loci. Shaded areas show the shared alleles for both pairs of samples. H- Houlden *et al*'s (1996) primers, C- Cahill's (2001) primers.

(a) CM3 and 16

		Phc 1		*Phc* 2		*Phc* 4		*Phc* 11		*Phc* 13		*Phc* 25	
H	CM3	104	104	200	192	109	107	179	159	117	111	121	121
	CM16	104	104	200	192	109	107	179	159	117	111	121	121

		K2.1		K10.1		*Pcv* 25.2		*Pcv* 6.1		*Pcv* 6.3		*Pcv* 7.1		*Pcv* 21.3		*Pcv* 32	
C	CM3	158	168	127	138	164	164	208	208	292	301	268	268	149	163	183	190
	CM16	158	174	127	138	164	164	208	227	295	301	268	268	149	149	181	190

(b) CM 11 and 20

		Phc 1		*Phc* 2		*Phc* 4		*Phc* 11		*Phc* 13		*Phc* 25	
H	CM11	104	102	192	192	107	107	171	171	117	111	129	121
	CM20	104	102	192	192	107	107	171	171	117	111	129	121

		K2.1		K10.1		*Pcv* 25.2		*Pcv* 6.1		*Pcv* 6.3		*Pcv* 7.1		*Pcv* 21.3		*Pcv* 32	
C	CM11	158	172	138	138	164	172	208	227	292	292	268	268	144	149	183	190
	CM20	158	172	138	138	164	172	208	208	295	295	263	268	144	144	183	190

3.4 Faecal DNA amplification

The eight microsatellite primer sets of Cahill (2001), K2.1, K10.1, *Pcv* 6.3, 6.1, 7.1, 21.3, 25.2 and 32, were used to amplify tissue and faecal DNA samples obtained from koalas 'Newman' and 'Lyn', to determine if 2 µl of faecal DNA per PCR reaction was sufficient to detect microsatellite alleles. Each faecal DNA sample was amplified in triplicate to ensure all results were reproducible and accurate as well as to prevent errors associated with stochastic sampling such as false alleles and allelic dropout. Faecal DNA amplification was deemed to be successful when the allele bands in the faecal replicates matched the alleles bands in the tissue sample. Successful faecal DNA amplification was obtained for each locus. Figures 3.16- 3.22 show the result obtained for these reactions.

The faecal DNA samples all amplified and produced identical banding patterns to the tissue samples. No false alleles or allelic dropout was detected. Non-specific bands, not present in the tissue reaction were detected in most faecal reactions. However these bands did not overlap the position of the alleles. The negative controls were free of contamination.

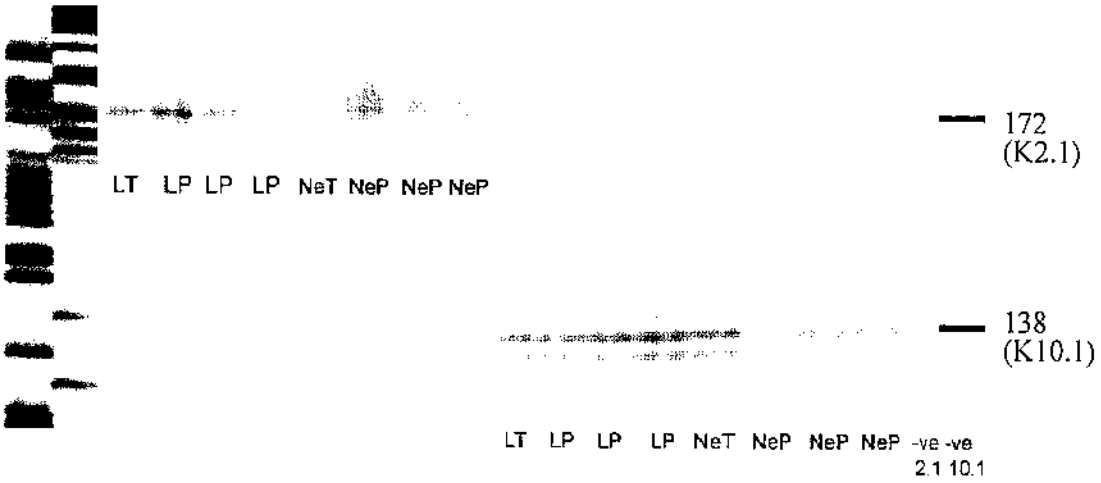


Figure 3.16 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for K2.1 and K10.1. A and G =M13mp18 DNA marker. Allele sizes are in base pairs

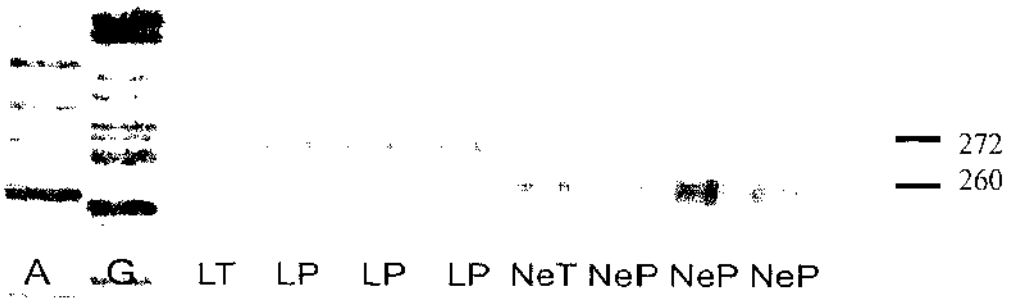


Figure 3.17 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 7.1. A and G =M13mp18 DNA marker. Allele sizes are in base pairs

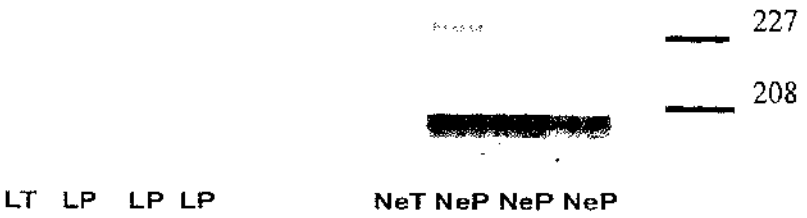


Figure 3.18 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 6.1. Allele sizes are in base pairs. Note the poor intensity of the amplified samples from Lyn, particularly the third pellet replicate. Both alleles are present and can be seen with the aid of a light box.

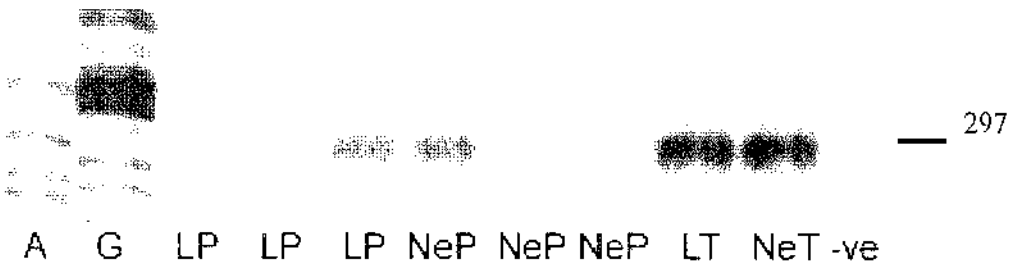


Figure 3.19 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 6.3. A and G =M13mp18 DNA marker. Allele sizes are in base pairs.

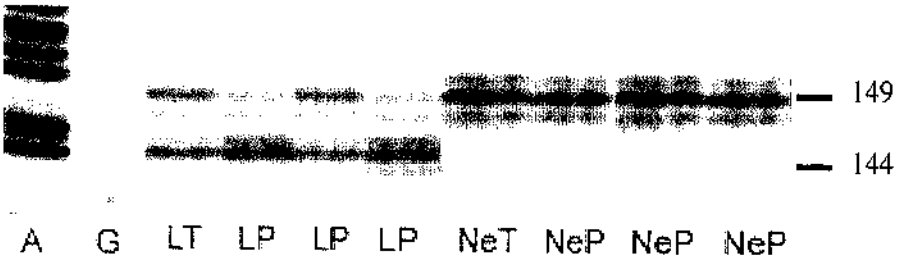


Figure 3.20 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 21.3. A and G =M13mp18 DNA marker. Allele sizes are in base pairs.

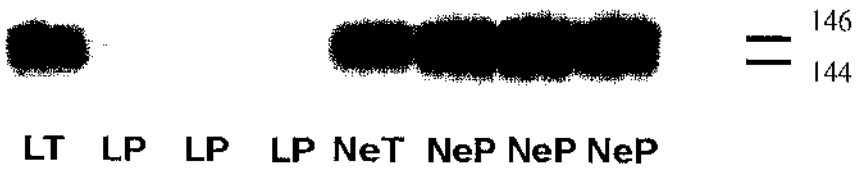


Figure 3.21 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 25.2. Allele sizes are in base pairs.

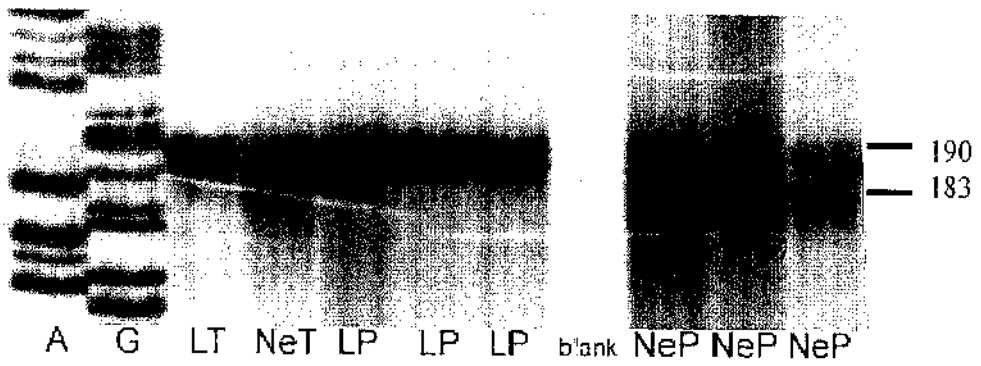


Figure 3.22 Autoradiograph showing microsatellite patterns derived from tissue (T) and faecal DNA (P) of Newman (Ne) and Lyn (L) for *Pcv* 32. A and G =M13mp18 DNA marker. Allele sizes are in base pairs.

3.5 In-house blind trial

Nine unknown faecal DNA samples were screened in a blind trial using primer sets K2.1, K10.1, *Pcv* 6.3, 6.1, 7.1, 21.3, 25.2, and 32. Each sample was amplified in triplicate to ensure all results were reproducible and accurate and to check for false alleles and allelic dropout. All unknown faecal DNA extracts contained amplifiable nuclear DNA for at least one of three replicates for all loci. The genotypes obtained for each unknown faecal DNA sample are recorded in Table 3.5. The negative controls for all reactions showed no contamination.

Failed PCR reactions occurred for one or two replicates of samples AM1, AM2, AM3, AL2, AL3 and M1 at loci *Pcv* 6.3, 7.1 and 21.3. Allelic dropout occurred in single replicates of samples AM2, at loci *Pcv* 6.3 and 21.3, and AL2 and M3 at locus *Pcv* 21.3. No allelic dropout or failed reactions occurred for samples AL2 and M2 at any locus. Overall, for the eight loci examined, allelic dropout occurred in about 1.85 % of faecal DNA amplification and 4.63% of PCR reactions failed. These results are summarised in Table 3.6.

Genotypes were accepted as accurate only if they matched in all replicates or if only one replicate of a sample yielding a heterozygous genotype showed dropout of one allele. In such cases the heterozygous genotype was accepted. Additional PCR reactions were not performed to confirm the genotypes for failed reactions, because the initial amount of unknown sample required for analysis was inaccurately dispensed. despite being performed by experienced lab workers familiar with the equipment.

Table 3. 5 Microsatellite genotype results for the unknown faecal samples used for the in house 'blind' trial. Triplicate reactions were performed on each sample for all loci.

	Allele size in base pairs for each locus							
Sample	K 2.1	K 10.1	*Pcv* 25.2	*Pcv* 6.1	*Pcv* 6.3	*Pcv* 7.1	*Pcv* 21.3	*Pcv* 32
	172 172	139 139	164 166	208 208	297 297	* *	* *	183 189
Am1	172 172	139 139	164 166	208 208	297 297	272 272	150 150	183 189
	172 172	139 139	164 166	208 208	297 297	272 272	150 150	183 189
	158 168	127 139	164 172	208 228	297 297	* *	144 150	181 189
Am2	158 168	127 139	164 172	208 228	297 297	272 272	144 *	181 189
	158 168	127 139	164 172	208 *	297 297	272 272	144 150	181 189
	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
Am3	172 172	139 139	164 164	208 228	297 297	* *	144 150	189 189
	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
AL1	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
	172 172	139 139	164 164	208 228	297 297	262 262	144 150	189 189
	158 168	127 139	164 172	208 228	297 297	272 272	* 150	181 189
AL2	158 168	127 139	164 172	208 228	297 297	* *	* *	181 189
	158 168	127 139	164 172	208 228	297 297	272 272	144 150	181 189
	158 172	139 139	164 172	208 208	285 301	* *	144 150	183 189
AL3	158 172	139 139	164 172	208 208	285 301	268 268	144 150	183 189
	158 172	139 139	164 172	208 208	285 301	268 268	* *	183 189
	158 174	139 139	164 172	206 228	295 295	272 272	144 144	189 189
M1	158 174	139 139	164 172	206 228	* *	272 272	144 144	189 189
	158 174	139 139	164 172	206 228	* *	272 272	144 144	189 189
	158 158	139 139	172 172	208 208	285 293	268 268	150 150	181 189
M2	158 158	139 139	172 172	208 208	285 293	268 268	150 150	181 189
	158 158	139 139	172 172	208 208	285 293	268 268	150 150	181 189
	158 168	127 139	164 172	208 228	295 295	272 272	144 150	181 189
M3	158 168	127 139	164 172	208 228	295 295	272 272	144 *	181 189
	158 168	127 139	164 172	208 228	295 295	272 272	144 150	181 189

Single asterix (*) Indicates allelic dropout.
Double asterix (* *) Indicates a failed PCR reaction.

Table 3.6 Proportion of correct genotypes, failed PCR reactions and allelic dropouts per locus, for in-house blind trial faecal samples.

Locus	Correct Genotype[A]	Failed reactions[B]	Allelic dropout[C]
K 2.1	27	0	0
K10.1	27	0	0
Pcv 25.2	27	0	0
Pcv 6.1	26	0	1
Pcv 6.3	22	2	0
Pcv 7.1	22	5	0
Pcv 21.3	21	3	3
Pcv 32	27	0	0
Overall frequency	92.13%	4.63%	1.85%

n- total number of reactions performed per locus (n=27).
[A]-Number of faecal PCR reactions where microsatellite patterns matched a tissue profile.
[B]- Number of faecal PCR reactions which did not produce a product.
[C]- Number of faecal PCR reactions that showed an allelic dropout i.e. one replicate was homozygous and the rest were heterozygous.

The sizes of the microsatellites obtained for each unknown sample were compared to the genetic database (Table 3.2) and a preliminary identification assigned to each sample. Eight individuals were unequivocally identified based on a genetic match with Table 3.2. The ninth individual was tentatively assigned the identity of Nathan, as it matched this individual and no other, at seven of the eight loci. This identity proved correct.
These results are summarized in table 3.7

Table 3.7 Probable and true identities of unknown faecal samples amplified during the in-house blind trial.

Sample	Number of loci matched	Probable identity	True identity
Am1	8	Newman	Newman
Am2	8	Nathan	Nathan
Am3	8	Lyn	Lyn
AL1	8	Lyn	Lyn
AL2	8	Nathan	Nathan
AL3	8	Fran	Fran
M1	8	Breyk	Breyk
M2	8	Marlee	Marlee
M3	7	Nathan (?)	Nathan

Allelic dropout reduces the probability of identifying individuals within a population. Therefore a revised PI was calculated for each sample used in the blind trial. The probability was corrected for the loci which showed allelic dropout in Table 3.5. The probability of identity varied between 3.11×10^{-5} to 5.12×10^{-4} for unrelated individuals and 7.00×10^{-4} to 4.18×10^{-3} for siblings. The average PI and PI_{sibs} for the blind trial was found to be 9.07×10^{-5} and 8.98×10^{-4} respectively.

A summary of the results is shown in Table 3.8.

Table 3.8 Probability of identity for each sample corrected for allelic dropout. The PI using 8 loci is 3.11×10^{-5} for unrelated individuals and 7.00×10^{-4} for siblings.

Sample	No. Loci*	Corrected PI	Corrected PI_{sibs}
Am1	8	3.11×10^{-5}	7.00×10^{-4}
Am2	6	5.12×10^{-4}	4.18×10^{-3}
Am3	8	3.11×10^{-5}	7.00×10^{-4}
AL1	8	3.11×10^{-5}	7.00×10^{-4}
AL2	7	8.70×10^{-5}	1.60×10^{-3}
AL3	8	3.11×10^{-5}	7.00×10^{-4}
M1	8	3.11×10^{-5}	7.00×10^{-4}
M2	8	3.11×10^{-5}	7.00×10^{-4}
M3	7	8.70×10^{-5}	1.60×10^{-3}
Average	-	9.07×10^{-5}	8.98×10^{-4}

* The number of loci which did not show allelic dropout. This number of loci were used to obtain a corrected probability of identity for each sample.

3.6 Analysis of genotypic data for tissue samples

A large amount of allelic data was generated for eight polymorphic loci (Cahill, 2001) using tissue samples during this study, allowing a brief statistical analysis of genetic variation within and between the four populations, Campbelltown, Bargo, Colo Heights and Heathcote, to be carried out.

A total of fifty-eight alleles were identified for eight microsatellite loci. Between 5 and 10 alleles were found per locus, the majority of which were shared between at least five individuals and between most populations of koalas. A total of 21 unique alleles was detected, 10 were found in the Bargo, Colo Heights and Heathcote populations and 11 within the Campbelltown population. The size of the most of these alleles at each locus are separated by 2-5bp. A summary of these unique alleles can be found in Table 3.9. No null alleles were detected for the Campbelltown population.

Table 3.9 Unique alleles found in the Campbelltown, Colo Heights, Bargo and Heathcote koala populations.

Population	Locus	Allele sizes (bp)
Campbelltown	K10.1	129
	Pcv-6.1	212, 230, 232
	Pcv-6.3	281, 283, 297
	Pcv-7.1	276, 280
	Pcv-21.3	142, 162
Colo Heights	K10.1	125
	Pcv-25.2	168, 170
	Pcv-32	185
Bargo	K2.1	156
	Pcv-6.1	204
	Pcv-21.3	152, 156
Heathcote	*Pcv*-6.1	216
	Pcv-7.1	252
	Pcv-32	195

The heterozygosity values for each group of koalas are shown in Table 3.10. The average observed heterozygosity (H_O) for Campbelltown was 0.473 and the heterozygosity expected under Hardy Weinberg equilibrium (H_E) 0.632. The other three populations analysed had a heterozygosity of between 0.400 and 0.670. However the values for Colo Heights, Bargo and Heathcoate do not reflect the true heterozygosity of these populations due to small sample sizes ($n < 8$).

The results for the exact tests for conformity to Hardy Weinberg equilibrium for Campbelltown, Colo Heights, Bargo and Heathcote populations at each locus are located in Appendix six and are summarized in table 3.11. There was a significant departure from Hardy Weinberg equilibrium for the Campbelltown population at all loci ($p<0.05$). However, all the other populations generally conformed to Hardy Weinberg expectations.

Table 3.10 Average heterozygosity values for four koala populations.

Population	Population Size	Direct count H_O	Expected under HWbg H_E
Campbelltown	46	0.462	0.627
Colo Heights	6	0.450	0.570
Bargo	7	0.436	0.662
Heathcote	4	0.500	0.590

Table 3.11 Probability values for deviations from Hardy Weinberg equilibrium for eight polymorphic microsatellite loci for four koala populations. The probability values show significant deviation from Hardy Weinberg when $P < 0.05$.

	Locus							
Population	K 2.1	K 10.1	*Pcv* 25.2	*Pcv* 6.1	*Pcv* 6.3	*Pcv* 7.1	*Pcv* 21.3	*Pcv* 32
Campbelltown	0.0009	0.005	0.003	0.005	0	0	0	0
Colo Heights	0.6145	0.0524	0.6251	—*	0.5484	0.5484	0.4266	0.0016
Bargo	0.8184	1	0.0036	0.0135	0.0221	0.7227	0.003	0.088
Heathcote	0.1489	1	1	0.0299	1	1	1	0.3254

* No P- value was obtained as this population was homozygous for this locus.

The F-statistics, which measure the level of inbreeding within and between populations, were measured for the Campbelltown and other three populations. The average results are given in Table 3.12. The high average score for mean F_{ST} (0.133) indicates that Campbelltown koalas are genetically different from koalas found at Colo Heights, Bargo and Heathcote. The highly positive scores for F_{IS} and F_{IT} indicate that the populations are inbred. Due to small sample sizes these statistics are not true values but do provide an indication of the degree of differentiation between koala populations in and around Southern Sydney.

Table 3.12 Average F-statistics for Campbelltown, Colo Heights, Bargo and Heathcote populations for eight microsatellite loci.

Locus	F_{IS}^	F_{IT}*	F_{ST}**
K2.1	0.198	0.330	0.164
K10.1	0.260	0.408	0.199
Pcv-25.2	0.179	0.276	0.118
Pcv-6.1	0.467	0.539	0.136
Pcv-6.3	0.345	0.474	0.198
Pcv-7.1	-0.024	0.041	0.063
Pcv-21.3	0.265	0.334	0.095
Pcv-32	0.343	0.399	0.086
Average	0.254	0.350	0.133

^F_{IS} - A measure of the deviation from Hardy Weinberg proportions within subpopulations.

*F_{IT} - A measure of the deviation from Hardy Weinberg proportions in the total population.

**F_{ST} - Wright's inbreeding coefficient (fixation index), a measure of the genetic differentiation over subpopulations relative to the differentiation in the total population.

The average migration rate between the four populations was determined using Wrights (1969) equation (section 2.2.16) and the F statistics in Table 3.12. The migration rate was found to be 1.63 indicating a degree of gene flow is occurring between all four populations sampled in this study.

4.0 Discussion

4.1 Faecal pellet collection and preservation

Although koala pellets are reasonably easy to identify, natural differences in the size and shape of pellets can occasionally make the identification and collection of koala pellets difficult. Approximately 9% of a koalas daily faecal pellet production will be found within 1m of the occupied tree (Ellis *et al.*, 1998). Therefore, if one koala deposits 174 pellets per day, up to 15 pellets might be sampled from this individual at one location (Ellis *et al.*, 1998). The collection of pellets is also dependant on their physical condition i.e. age and exposure to weather and the collector's experience in identifying a patina, as these factors govern successful DNA extractions (Flagstadd *et al.*, 1999). Although a patina is advantageous, the physical appearance of the pellets should not be used as a definitive guide to the presence of DNA, as pellets which have dried quickly in warm, dry climates are often better preserved than pellets from wet environments, which may appear fresh but can degrade quickly (Curtin *et al*, 2002; Ward 2002).

Fresh pellets are preferable for DNA work. However, pellets can be stored and the cells adhering to their surface preserved using varying conditions for extended periods. The optimal storage method found in this study was room temperature storage after spraying with 70% ethanol. Alternatively storage at –70 °C also preserves cells. These results confirm the findings of Wasser *et al.* (1997) who found that DNA could be recovered and amplified after six months from pellets stored at –70 °C, and at room temp after spraying with 70% ethanol. The findings of Wasser *et al.* (1997) for storage at 4°C and room temperature were also confirmed as little DNA could be recovered from pellets stored at 4°C or at room temperature after six months. The techniques described in this thesis should not generate erroneous results when used on dry pellets or those collected in arid environments. However, if pellets are collected when wet or freshly deposited, cells on the surface of the pellets may be shed onto any surface they contact. Therefore, to prevent the loss of these cells, individual pellets should first be sprayed with 70% ethanol to promote the preservation of the DNA, then individually wrapped in a small amount of paper. Each pellet should also be collected individually to avoid transferring DNA between pellet samples.

Koalas defecate between 1800hours and midnight (Ellis *et al.*, 1998). Therefore, pellet collection should be conducted from midday to midafternoon, to allow early morning dew to evaporate and allow fresh pellets a chance to dry. It is not recommended that pellets be collected after fire or heavy rain, as there is little chance of DNA surviving these conditions.

As with the collection process, strict standards for the transportation of koala pellets must be met, to avoid damage to the pellets before DNA extraction. This particularly applies to pellets collected in plastic or paper sample bags, which offer little to no protection during transport. Where possible, pellets should be collected in small specimen jars, and sample bags should be transported inside a padded envelope. The guidelines for the collection and transport of koala faecal pellets can be found in Appendix 6.

4.2 DNA extraction from pellets

The two methods of collecting intestinal cells from the outer surface of the pellets are surface scraping and surface washing. The dry, compact nature of koala faecal pellets facilitates the washing of the epithelial cells from the pellets outer surface. The nature of the pellets allows the reliable replication of the surface wash method on a variety of pellets of different shapes and sizes. Therefore this method was chosen to collect cells from the surface of koala pellets prior to DNA extraction.

Kohn *et al.* (1995) suggest carrying out multiple extracts on the one pellet and pooling the extracts in order to increase the potential DNA yield. This strategy was successful and results show that performing multiple surface washes on each pellet and pooling the extractions during the final stages of the extraction procedure provides a high yield of DNA, which can be visualised and quantified on an agarose gel (Figure 3.3a and 3.3 b). As well as multiple surface washes, an additional extraction is also required for wet pellets. Cells on the surface of these pellets can be transferred by contact and dried onto paper. As a result, multiple surface washes will not recover the maximum amount of DNA present on the sample. In fact, DNA extractions performed on various types of paper which had been wrapped around wet pellets show that a significant amount of DNA can be recovered from paper and that there is no significant difference between the types of paper used for pellet collection (Figure 3.5). Therefore, a DNA extraction

should be carried out on the paper in conjunction with the surface washes and the paper and pellet extracts pooled to ensure the maximum amount of DNA is recovered from each pellet sample.

Many protocols exist for the extraction of DNA from faeces (Deuter *et al.*, 1995; Foran *et al.*, 1997; Wasser *et al.*, 1997; Flagstadd *et al.*, 1999; Banks *et al.*, 2000). Many of these protocols are loosely based on one another and in most cases require an overnight incubation of the stool sample in a lysis buffer. Though each of the existing protocols have been successful, the amount of time involved for individual extractions is not favorable when processing a large number of samples. Therefore a procedure which extracts as much DNA as possible from each sample, removes potential PCR inhibitors and is quick and simple to follow (Reed *et al.*, 1997) was required.

A Qiagen kit (QIAGEN Pty. Ltd) and manufacturer's protocol for extracting DNA from tissue and blood was successfully used by Wasser *et al.*, (1997) and Flagstadd *et al.*, (1999) to obtain faecal DNA which could be used in PCR reactions. This method of obtaining DNA is rapid and easy to follow, but does not remove PCR inhibitors. QIAGEN solved this problem by developing the QIAamp DNA stool mini kit. The protocol for this kit is similar to the tissue kits but includes additional steps to remove PCR inhibitors. Clarke (2000) used this kit and showed that the QIAamp DNA stool mini kit could be used to provide a quick and simple method of purifying DNA from faeces.

To confirm the findings of Clarke (2000), the surface of an old koala pellet was spiked with water rat DNA and the DNA extracted using the QIAamp DNA stool mini kit and protocol for the isolation of DNA for human analysis. Although the amount of water rat DNA recovered from the surface of the pellets was not sufficient to visualise on an agarose gel, PCR analysis, using microsatellites, confirmed the success of the DNA extraction procedure. The lack of any other bands on the gel also confirmed that the extraction procedure removed inhibitors present on the faeces.

The recovery of DNA was improved by adjusting the incubation time of the surface washes in lysis buffer to at least 30 min, a considerably longer time than the five minutes used to recover the spiked water rat DNA. The initial time of five minutes was

adequate to recover the spiked DNA as no cell lysis was required. However, the surface washes contained an unknown number of intestinal cells which had dried to the pellets under a layer of mucus. Therefore increasing the lysis time to at least 30 min ensured that the mucus layer was broken down and all cells in the washes lysed.

A further improvement to the Qiagen protocol involved the final elution of the DNA from the silica-gel membrane in the spin column. The manufacturer recommends incubating the DNA with 200 µl of elution buffer, for one minute before elution. However, this timing was insufficient and did not release the koala DNA which had bound to the spin column. I found that a 5 min incubation before elution, followed by two elutions, increases the elution efficiency and recovered a greater yield of DNA.

Also of concern during extraction was the fragility of the pellets. The koala pellet easily absorbed moisture and in a small amount of time became brittle. Therefore the wash procedure needed to be completed in a short period of time (≤ 1 minute). Initial washes were easily accomplished with no observable damage to the pellet and in most cases both surface washes were readily performed. However, if the pellet surface was damaged, either before or during the surface washes procedure, then the wash buffer was absorbed into the pellet. If the damage happened during the wash process then it was possible that some of the intestinal cells washed into the buffer were absorbed into the pellet, thus decreasing the potential yield of DNA. Damage to the pellet also resulted in pellet debris entering the extracts. Although some debris is initially present in any faecal DNA extraction, in the cases where the pellet was damaged, larger amounts of debris were able to enter the wash buffer. This internal debris, consisting of digested plants and other contaminants, might affect later applications of the DNA if not fully removed during the extraction procedure. Therefore any pellet with a damaged surface could not be used.

The electrophoresis of pellet and tissue DNA extracts (Figure 3.3a and b) show a clear band for all samples. There was no indication of contamination by RNA or other contaminants, such as bacteria or plant matter. This confirms that the modified Qiagen method of DNA extraction and purification are highly reliable for the koala. However, the QIAamp DNA stool kit would not be recommended for large sampling, as the manufacturer does not supply its inhibitor remover, inhibitEX tablets, as a separate

component. Thus to process more than 25 samples, more than one kit would be required. This wastes most of the buffers supplied in individual kits and increases costs associated with faecal DNA work. Therefore, for bulk sampling, a different method of removing the inhibitors should be found. A solution to this problem might lie in the use of Dueter *et al.*'s (1995) stool lysis buffer. This buffer contains a polysaccharide matrix to bind and remove PCR inhibitors from faecal DNA extractions and has been shown to work in conjunction with QIAGEN kit buffers by Banks *et al.* (2002).

The need to modify the initial and final stages of the QIAamp DNA stool kit protocol has been confirmed by Morin *et al,* (2001), using chimpanzee faeces, who verify the optimal incubation time for buffer ASL of between 30-60 min. However, where I recommend a two-step elution with five minute incubations, Morin *et al,* (2001) propose using a single elution carried out over 20-30 min. Overall, Morin *et al,* (2001) obtained a higher yield of DNA (between 38- 510 ng in 200 µl) using the 20-30 min incubation. However, when the nature and size of the faecal samples are taken into consideration, surface washes of a koala pellet vs. 100 mg homogenized chimpanzee faeces, the difference in yield is not significant, as the DNA yield varies with the size of the faecal sample as well as between species and individuals (Taberlet and Luikart, 1999; Goossens *et al.*, 2000). However the different methods for elution and the DNA yields obtained indicate that the elution step of the QIAamp DNA stool kit can be altered at the researcher's discretion to fit the particular study.

4.3 Mitochondrial DNA analyses

The fragment chosen for amplification was located in the 5' end of the mtDNA control region. This region of the mitochondrial genome is highly conserved, but varies in size between phyla and orders by a factor of 1.3 (Brown, 1985; Randi, 2000). The primers used in this study to amplify mitochondrial DNA (mt15996L and mt16502H) were originally designed to amplify the tRNA gene flanking the control region and the central conserved domain of the mtDNA control region in the opossum (Houlden *et al.*, 1999). Houlden *et al.* (1999) found these primers to be heterologous and the fragments generated by these primers in the koala to differ from the opossum, wallaby and other mammals by ~400bp. Therefore, these heterologous primers are ideal to determine the species of origin for faecal DNA samples.

If the DNA extracted from surface washes of koala faecal pellets was too degraded to amplify, or was not present in high copy number, no results would be produced in a mitochondrial PCR assay. Thus mtDNA analysis provided a simple and rapid indication of the success of both the faecal DNA extraction and pellet collection process. Positive control tissue samples from known animals, amplified in conjunction with faecal DNA extracts, provides a visual reference of the success of each faecal DNA extraction. Any faecal DNA extract producing mtDNA bands which have not migrated the same distance as the positive control, or which show no result, can be quickly excluded from future analyses.

Electrophoresis of the mtDNA PCR reactions from faecal DNA showed that the DNA was likely to be that of the koala as the amplified tissue and faecal DNA fragments migrated with the same size of ~832bp, close to the ~850 bp found by Houlden *et al* (1999) for koala DNA. Although sequencing the mtDNA PCR fragments would provide a definitive identification of the species from which the faecal sample was obtained, the presence of the ~832 bp fragment in all samples analysed during this study was a satisfactory indicator that the origin of the faeces was koala.

The absence of any other bands in the samples and negative control further confirms the purity of the pellet samples and the validity of the koala pellet DNA extraction method.

4.4 Microsatellite evaluation

Repeated attempts to optimize the six koala specific primers of Houlden *et al.* (1996) proved unsuccessful. Adjusting the concentrations of PCR reagents and cycling parameters had little or no effect. Even the use of a high fidelity *Taq* polymerase failed to optimize results. Other inter-laboratory differences e.g. air conditioning, humidity, PCR machines and sequencing apparatus may have also contributed to the poor results obtained by this study (per comm.; Dr Mark Eldridge).

Sizing of DNA fragments is accomplished by comparing the electrophoresed distance of coelectrophoresed fragments to a size standard. However, comparative fragment sizes are not always accurate to the nucleotide, and can vary between PCR reactions for the same samples (Schwengel *et al*, 1994; Haberl and Tautz, 1999). The problem of accurate sizing can also be compounded by the size standard used to measure the

alleles, as different markers possess different size scales, and by the appearance of stutter bands surrounding the allele (Haberl and Tautz, 1999). Usually the most intense band is the first band within the stutter array and this band is scored as the allele (Haberl and Tautz, 1999). However in some reactions, slippage events may cause a stutter band to be amplified in similar or greater proportions to the allele (Taberlet *et al*, 1996). These stutter bands may be amplified 1-2bp above or below the primary allele (Scribner and Pearce, 2000), therefore care must be taken to note the position of the most intense band within the array. The difference in allele sizes obtained for samples CM1, 2 and 3 at loc *Phc*- 2, 4 and 11 for this study compared to the preliminary database in Appendix 4 (2-3 base pairs) is most likely a result of a slippage event which has amplified a stutter band preferentially to the actual allele as the same marker was used to size the samples for the preliminary database.

In contrast, PCR reactions using Cahill's (2001) primers were relatively easy to optimise and alleles could be positively identified and consistently sized with few problems. The exceptions were loci K10.1 and 6.1. Both loci amplified extra bands which interfered with the identification and sizing of the true alleles. The 133bp band at locus K10.1 was amplified in both homozygous and heterozygous individuals, in similar proportions to the actual alleles in some individuals. Reducing the amount of primer used in PCR easily removed this band, indicating that it was most likely a stutter band amplified preferentially to the allele.

A 222bp band was detected in only a few individuals at locus *Pcv* 6.1. The band was a sufficient distance from the allele (208bp) amplified in each sample and close in size to another allele amplified in other samples (228bp) to indicate that a secondary recognition site for this primer existed in some samples, i.e. an allele was present at this location. Contamination of the samples was unlikely to be the cause of the extra band as it appeared in only a select few samples and the negative controls showed no abnormalities. In most cases where a single band was produced it was considerably brighter than the other allele. This indicated the main allele was preferentially amplified during PCR, and there was little repeat tract instability to form stutter bands. However, the stutter bands may have been present in all reactions, but in insignificant quantities, resulting in a single intense band appearing on the gels. This was confirmed when the reactions for the samples containing this single 222bp band were repeated, as very faint

stutter bands were observed for some samples. Although this band was considered as an allele due to the observed stutter effect in some reactions, it will be necessary to sequence this band to confirm this band is an actual allele.

4.5 Microsatellite Genotyping

In studies requiring individual identification it is important to approximate the maximum and minimum number of loci necessary to distinguish between different individuals (Taberlet *et al.*, 1999; Waits *et al.*, 2001). The statistics most commonly used are the probability of identity (PI), the probability that two individuals drawn at random from a population will have the same genotype at multiple loci, and the probability of identifying full siblings (PI_{sibs}) (Mills *et al*, 2000; Waits *et al*, 2001). Waits *et al.* (2001) computed PI for different heterozygosities to provide approximate guidelines as to the number of loci required to identify individuals. The guidelines are based on allele frequencies for various numbers of loci for different heterozygosities. A theoretical PI_{sibs} and PI of between 1×10^{-2} and 1×10^{-4} respectively is necessary for identifying individuals within wild animal populations (Waits *et al.*, 2001), these are similar values to the theoretical PI and PI_{sibs} calculated for the preliminary genetic database for Campbelltown koalas (1.41×10^{-3} and 1.57×10^{-2} respectively, see Appendix 4). Therefore using the guidelines of Waits et al (2001) and the heterozygosity for Campbelltown koalas (~0.6), the number of loci required to identify individual koalas was determined to be between 7-14. This range was confirmed when analysing the genotypes of individuals CM3 and CM16, and CM11 and CM20. These pairs of individuals showed identical genotypes when analysed using Houlden *et al.*'s (1996a) six microsatellite loci, and show different genotypes when the number of loci analysed is increased to 14 (Table 3.4). However, these samples are also shown to be individuals with six of Cahill's (2001) loci (Table 3.4), demonstrating that these loci are superior for determining individual genotypes.

The PI and PI_{sibs} using Cahill's (2001) eight primer sets was estimated to be 3.17×10^{-5} and 8.08×10^{-4} respectively, an order of magnitude higher than the estimation made using Houlden *et al.*'s (1996a) primers. The calculated value is sufficiently low that the risk of misidentifying individuals is small. However, data is missing for some genotypes for some loci. Therefore the true value for PI and PI_{sibs} is likely to be much less for the Campbelltown population.

Unique genotypes were determined for 61 individuals, for at least seven of eight microsatellite loci, using primer sets K2.1, K10.1, *Pcv* 61, *Pcv* 6.3, *Pcv* 7.1, *Pcv* 21.3, *Pcv* 25.2 and *Pcv* 32. Few samples failed to amplify and those that failed were probably too dilute to be detected. In some cases repeating the reactions with greater amounts of DNA also failed to yield a result.

Allele sizes varied at all loci by 2bp and all loci contain dinucleotide repeat units. Therefore the cause of the variation is likely to be slippage mutation. This is consistent with the single stepwise mutational model of microsatellite evolution, which predicts that new alleles are generated through the addition or deletion of one repeat unit in an array. However, to confirm that slippage is the primary cause of allele size variation at the loci amplified in this study, it is necessary to sequence different sized alleles at each locus.

4.6 Faecal DNA analyses

Genotyping with microsatellites relies on the quality, accuracy and reproducibility of the result (Queller and Strassmann, 1993; Gerloff *et al.*, 1995; Haberl and Tautz, 2001). This is especially true for microsatellite amplification of DNA derived from faeces, in which the DNA being amplified is often partially degraded and present in low copy number (Gerloff *et al.*, 1995; Khon *et al.*, 1995; Flagstad *et al.*, 1999). The methods of DNA extraction from koala faecal pellets outlined in this study have been shown to generate high quality DNA, which can be amplified using heterologous mtDNA primers. However, mtDNA is present in cells in higher copy number than nuclear DNA (Gerloff *et al.*, 1995; Flagstad *et al.*, 1999), therefore the ability of microsatellite primers to detect and amplify loci in nuclear DNA in faecal extractions was also examined. These tests compared tissue and faecal DNA from two koalas and showed that the nuclear DNA in the faecal extracts was present in sufficient amounts to allow microsatellites to be amplified. The small number of PCR artifacts in the samples, which did not interfere with the allele position, showed that the faecal extracts were high quality and matching patterns for tissue and faecal DNA samples showed that faecal DNA could be reliably and accurately amplified.

The blind trial to identify individuals from their faecal DNA proved successful, with eight exact genotype matches made out of a possible nine matches, for all eight loci

examined. Each faecal DNA sample was amplified in triplicate to screen for non amplifying alleles and to ensure a PCR product and the correct genotype was obtained. 92.13% of reactions produced a genotype which matched a single genotype in the koala database for all three replicates (Table 3.8 and 3.9). This rate of success with faecal DNA amplification is greater than most in studies (Gerloff *et al.*, 1995; Reed *et al.*,1997; Taberlet *et al.*, 1997; Ernest *et al.*, 2000; Banks *et al.*, 2002), but similar to Flagstad *et al.* (1999) (93% for reindeer to 95% for sheep) and Morin *et al* (2001) (up to 97% for chimpanzees). The similar rate of success for faecal DNA amplification obtained by this study and Morin *et al* (2001) further confirms that the QIAamp DNA stool kit provides an easy and reliable method of extracting DNA from faeces.

The unknown faecal sample M3 was assigned the identity 'Nathan' as the genotype for Nathan matched the genotype for unknown sample M3 at seven loci. The faecal genotype did not match one of the other possible five individuals (see Table 2.1) whose faecal DNA extractions might have been used for the blind trial. The variation to the genotype occurred at locus *Pcv* 6.3 and the allele size in the faecal sample was found to be 295bp, a 2bp difference to the tissue genotype. This erroneous result has most likely been caused by slippage events occurring on degraded template, during PCR.

Three unknown samples (AM2, A12 and M3) in some reactions yielded only one of the two alleles. These non amplifying alleles are most likely due to random sampling when pipetting template DNA from a dilute extract. In some cases only one of the two chromosomes may have been pipetted for amplification (Gerloff *et al.*, 1995; Khon and Wayne, 1997; Taberlet *et al.*, 1999). Pipetting errors would also be the most likely cause of the ten failed PCR reactions, as most failed reactions were one of three replicates for different loci.

The fact that some PCR's do not work is a strong indication that the risk of incorrectly scoring a heterozygous individual as homozygous is high. However this risk can be moderated by increasing the number of replicates performed on single samples. Taberlet *et al.* (1996), propose that several replicates are required to reduce the error rate to below 1%. Therefore any samples which exhibit failed reactions or allelic dropout should be repeated and the number of replications increased from three to at least seven to ensure the correct genotype is obtained.

The number of PCR cycles commonly used by forensic anthropologists to amplify ancient DNA from bones is ≥45 (Hummel and Herrmann, 1994). Therefore, increasing the number of PCR cycles from 35 to 45 might also decrease the rate of error for faecal DNA microsatellite amplification. This would particularly apply to faecal samples which show poor results when amplified by mtDNA PCR. However, for most faecal DNA samples, 35 PCR cycles are sufficient for microsatellite analysis.

Despite the complications of allelic dropout and failed reactions, the average PI for the blind trial was 9.07×10^{-5} and 8.98×10^{-4} for unrelated individuals and full siblings respectively. This further confirms that the chance of misidentifying individuals from faecal genotypes is low even when using only six of Cahill's (2001) eight microsatellite loci.

The overall success of this blind trial indicates that the analysis of DNA obtained from the surface of koala faecal pellets could be used to identify individual koalas in the wild. However, due to unavoidable supply delays caused by the September 11, 2001 tragedy, and the repeated unsuccessful attempts at optimizing the PCR conditions of Houlden *et al.*'s (1996) microsatellite primers, this trial was only conducted on a limited number of known samples. Therefore to confirm the findings of the in-house blind trial, I recommend that the trial be extended to include unidentified pellet samples from koalas in the Campbelltown area.

4.7 Analysis of genotypic data for tissue samples

The increased number of loci available for the koala allowed a brief analysis of the genotypic data for 63 individuals to be carried out. The genomic frequencies for Heathcote, Colo Heights and Bargo, generally conform to the Hardy Weinberg model of equilibrium. This result indicates that these populations showed no influence from factors such as selective pressure, genetic drift and non-random mating. However, the Campbelltown population showed a significant deviation from Hardy Weinberg equilibrium ($p < 0.001$; Table 3.10). One explanation for this maybe the presence of a null allele. However, a comparison of the allelic inheritance pattern between parents and offspring for each locus suggests that null alleles are not present. This could be confirmed by sequencing the flanking region for homozygous individuals. A more likely explanation for the Campbelltown population deviating from Hardy Weinberg

equilibrium would be the relatedness of the individuals sampled. Several known females and their offspring have contributed much of the allelic data for this study (Ward 2002; R. Close, pers comm.). Therefore, the sample does not meet Hardy- Weinberg assumptions of a homogeneous population and this bias probably reduced the heterozygosity of the population to levels below those expected under Hardy Weinberg equilibrium.

The presence of unique alleles for five of Cahill's (2001) polymorphic loci (Table 3.8) indicates that genetic distinctions can be made between populations in Southern NSW. The degree of differentiation among and within populations can be measured using F-statistics (Wright, 1969). F statistics can be calculated using the relationship between heterozygosity and inbreeding. F_{ST} (fixation index) is a measure of the effect of population sub division on inbreeding. $F_{ST} = 0$ when populations are in Hardy-Weinberg equilibrium. The results obtained for the Campbelltown population (F_{ST} = 0.133) indicate that Campbelltown retains a moderate degree of population differentiation and is significantly genetically different to populations at Colo Heights, Bargo and Heathcote. This is consistent with the findings of Dr M.E. Montgomery (pers comm., 2001) who found significant levels of differentiation between Campbelltown and other coastal and inland koala populations of NSW.

The migration rate also suggests a level of gene flow, which is most likely due to male dispersal in koalas. The migration rate of 1.63 between each population is high and indicates that there is gene flow occurring between Southern Sydney koalas and those in the greater Sydney region. This is consistent with the findings of Ward (2002), who observed that koalas could migrate distances of at least 20 km.

The assumptions made in this study regarding the relationships between the Campbelltown, Colo Heights, Bargo and Heathcote populations can be used as the basis for future genetic studies of these populations. However, to obtain a better understanding of the relationships between these populations characterisation of a larger sample size for each population outside of the Campbelltown region is required.

4.8 Future research

The method for extracting DNA from koala faecal pellets, developed by this study, provides DNA free of major contaminants. This faecal DNA was used to generate genetic profiles, which when compared to the tissue profiles in the genetic database, allowed individuals within the Campbelltown population to be identified. This trial's success at identifying koalas from their faeces demonstrates that the analysis of microsatellite loci in koala faecal pellet DNA could be used to detect individuals in populations which are too sparsely distributed to allow the capture of individual koalas. A suitable case study would be the low density areas of New South Wales such as in the southeast forests (Allen, 2000).

Despite many community reports, the numbers of koalas in the southeast forests are unknown and few sightings of koalas have been made. However, the identification and collection of koala faecal pellets at the base of trees has provided evidence of a colony in the forests. These colonies face the possibility of extinction due to logging and extensive clearing of the forests for woodchipping (Allen, 2000; 2001). Therefore, the number of individuals in the area and their genetic status needs to be determined and molecular scatology techniques could be the solution to this problem.

Summary

The results detailed in this thesis show the achievement of the aim, to develop a method of identifying individual koalas from faecal DNA.

This study was successful in developing a quick and easy to follow faecal DNA extraction procedure, based on a commercial kit, which extracts DNA from each sample and removes potential PCR inhibitors. A simple change to the PCR inhibitor removal complex should make this procedure relatively inexpensive and allow large samples to be processed.

Although the six koala microsatellite primers *Phc*- 1, *Phc*- 2, *Phc*- 4, *Phc*- 112, *Phc*- 13 and *Phc*- 25, were successfully used in other studies and provided data for a preliminary genetic database for the Campbelltown population of koalas, these primers proved unsatisfactory in the present study. However, genetic profiles for 63 koalas, from three different populations, were successfully determined using the eight primer sets, K2.1, K10.1, *Pcv* 6.1, *Pcv* 6.3, *Pcv* 7.1, *Pcv* 21.3, *Pcv* 25.2 and *Pcv* 32. This genotypic data has been added to the existing koala genetic database providing a genetic profile for 14 microsatellite loci for at least 40 Campbelltown koalas.

An in–house blind trial successfully identified nine individuals from faecal DNA. The overall success rate of the faecal PCR reactions was 93.52% a result comparable with other studies. An extended blind trial, using unidentified pellet samples from koalas in the Campbelltown area, should further confirm these findings.

This thesis demonstrates that the analysis of microsatellite loci in koala faecal pellet DNA is a powerful tool which has the potential to detect individuals in populations which are too sparsely distributed to allow the capture of individual koalas.

Appendix 1: Concentration and fragment sizes of SPP-1 Phage DNA/ *Eco* R1 molecular weight marker

Table A1: Phage SPP-1 DNA size marker

Fragment No.	Length (Kb)	Log10 bp	Amount of DNA (ng)
1	8.58	3.93	97.5
2	7.43	3.87	84.5
3	6.1	3.79	69.3
4	4.9	3.69	55.7
5	3.64	3.56	41.4
6	2.8	3.45	31.8
7*	1.95	3.29	22.2
8*	1.88	3.27	21.4
9	1.52	3.18	17.3
10	1.48	3.17	16.8
11	1.16	3.06	13.2
12	0.99	3.00	11.3
13	0.71	2.85	8.1
14	0.49	2.69	5.6
15	0.36	2.56	4.1

* These markers usually travel as one band

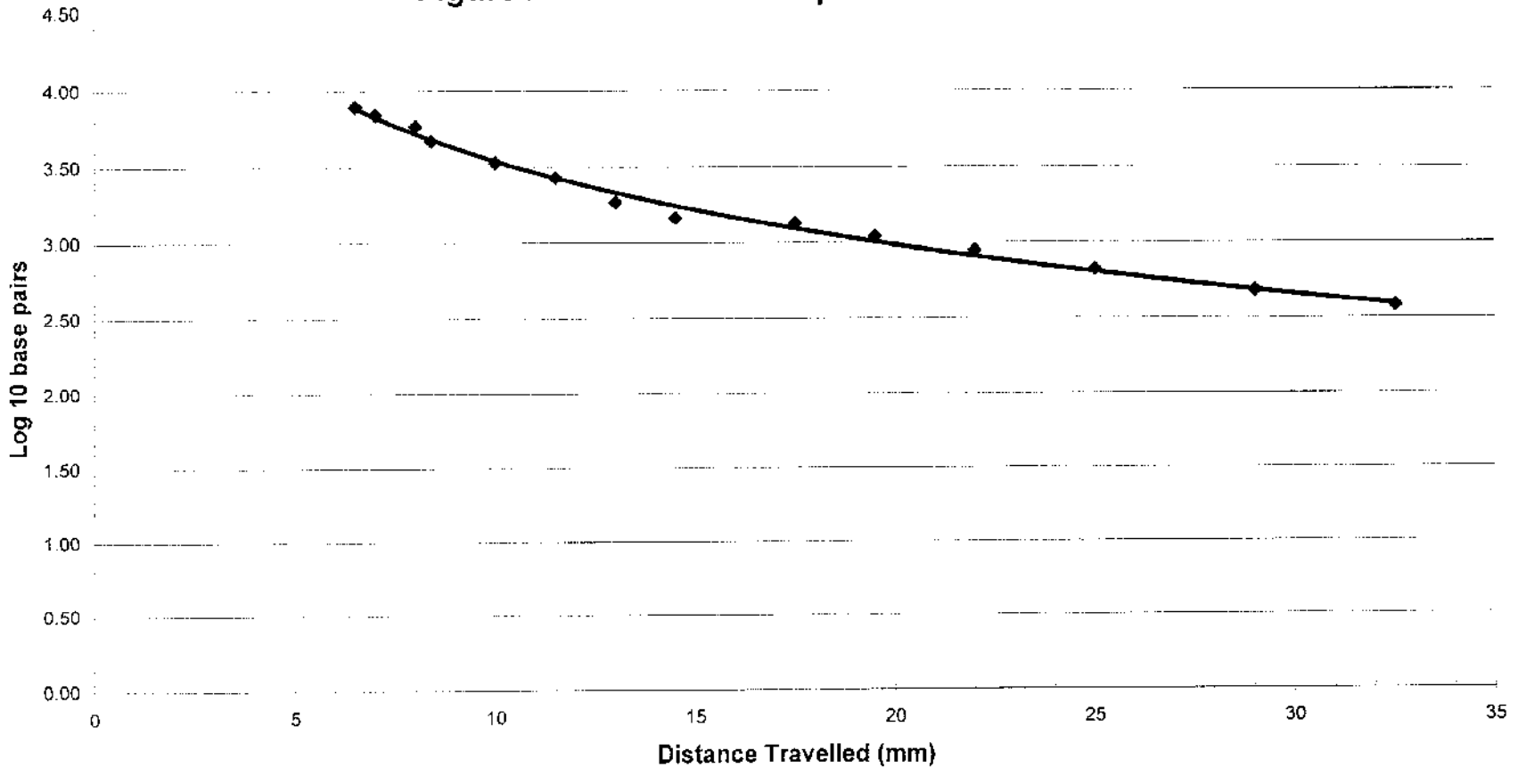
Appendix 2: SPP-1 marker calibration curve for sizing mitochondrial PCR fragments

Table A2: SPP-1 marker calibration curve measurements

Fragment No.	Distance Travelled (mm)	Log 10 bp
1	6.5	3.89
2	7	3.84
3	8	3.77
4	8.4	3.67
5	10	3.53
6	11.5	3.43
7*	13	3.27
8	14.5	3.16
9	17.5	3.12
10	19.5	3.04
11	22	2.94
12	25	2.82
13	29	2.68
14	32.5	2.58

* Bands 7 and 8 of the marker have travelled the same distance

Figure A1: SPP-1 marker plot

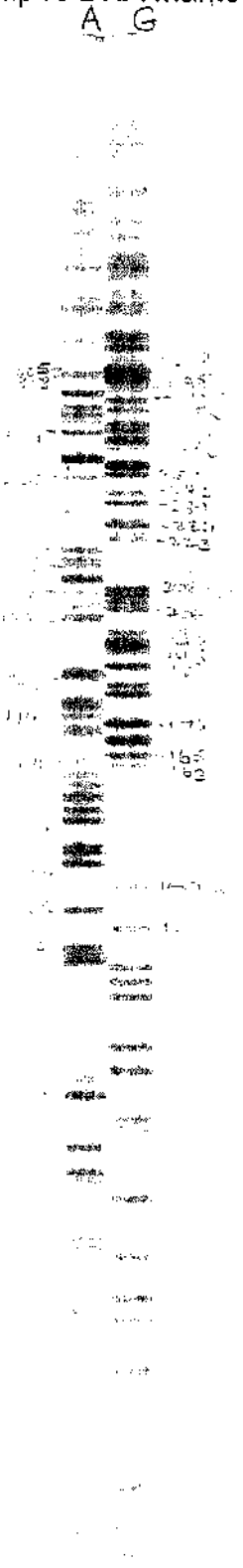


PLEASE NOTE

The greatest amount of care has been taken while scanning the following pages. The best possible results have been obtained.

Appendix 3: Fragment sizes of M13mp18 DNA markers, A and G

Figure A2: M13mp18 DNA marker, A and G



Appendix 4

Table A3: Preliminary genetic database for Campbelltown koalas

Assigned name	Name	*Phc* 1		Phc 2		Phc 4		Phc 11		Phc 13		Phc 25	
CM1	Bluey	102	102	200	176	107	107	171	171	111	111	129	123
CM2	D96-2	102	98	192	192	109	107	179	171	111	111	129	129
CM3	Roger	104	104	200	192	109	107	179	159	117	111	121	121
CM4	D96-1	104	98	200	186	109	107	171	171	111	111	139	131
CM5	Harry	0	0	0	0	0	0	0	0	0	0	0	0
CM6	Mac	104	98	186	186	109	107	171	159	117	111	131	121
CM7	D96-3	102	102	186	176	109	107	175	159	111	111	139	129
CM8	D95-2	104	102	194	186	109	107	179	159	111	111	129	121
CM9	Hodge	104	102	194	186	109	107	159	159	117	111	129	121
CM10	Gary	104	98	192	186	109	107	179	179	117	111	131	121
CM11	Kevin	104	102	192	192	107	107	171	171	117	111	129	121
CM12	Kath	104	104	186	186	109	107	159	159	117	111	121	121
CM13	Gary	104	98	192	186	109	107	179	179	117	111	131	121
CM14	Fran	102	98	186	186	109	107	171	171	117	111	131	129
CM15	Sarah	104	98	186	186	107	107	179	171	117	111	131	121
CM16	D97-1	104	104	200	192	109	107	179	159	117	111	121	121
CM17	Gaylene	104	104	200	186	107	107	179	179	111	111	121	121
CM18	Steve	104	104	192	186	109	107	179	179	117	111	121	121
CM19	D94-1	102	102	194	186	107	107	171	159	117	111	129	129
CM20	D95-2	104	102	192	192	107	107	171	171	117	111	129	121
CM21	Amanda	104	104	192	186	107	107	179	171	117	111	121	121
CM22	Andrew	104	102	200	186	109	107	179	179	111	111	129	121
CM23	Jacob	104	104	192	186	109	107	179	159	117	117	121	121
CM24	Scott	98	98	186	186	109	107	179	159	117	111	131	121

Table A3 (cont): Preliminary genetic database for Campbelltown koalas

Assigned name	Name	*Phc* 1		Phc 2		Phc 4		Phc 11		Phc 13		Phc 25	
Hm710	D93-1	104	98	204	186	109	107	159	159	111	111	139	127
Y6163	Bridgett	104	98	200	200	107	107	159	159	111	111	143	127
95-1	95-1	98	98	204	200	109	107	171	171	111	111	121	121
95-2	Cascy	104	102	186	186	109	107	171	159	111	111	129	121
CM27	Orin	104	104	186	186	109	107	179	171	117	111	121	121
CM28	Leslie	104	104	0	0	109	107	179	161	117	111	121	121
CM29	Shirley	0	0	0	0	0	0	0	0	0	0	0	0
CM30	Alan	104	98	0	0	109	107	179	171	117	117	133	123
CM31	Ray	104	104	192	186	109	107	179	161	117	117	129	121
CM33	Sandy ii	102	98	192	186	107	107	171	171	111	111	139	129
CM34	Richard	104	102	200	186	107	107	171	161	111	111	121	121
CM35	???	104	102	200	192	109	107	173	161	117	111	145	127
CM36	Danae	104	102	0	0	109	107	179	177	111	111	129	121
CM37	Alby	104	102	192	192	109	107	171	161	117	111	129	121
CM38	Heath	104	98	192	192	107	107	171	161	117	111	123	123

Table A4: Allele sizes and heterozygousity for Houlden *et al.* 's (1996a) six loci.

Locus	No. alleles	Allele size in base pairs per locus									Ho	He
Phc 1	4	98	102	104	106						0.595	0.623
Phc 2	6	176	186	192	194	200	204				0.618	0.689
Phc 4	2	107	109								0.676	0.463
Phc11	7	159	161	171	173	175	177	179			0.568	0.729
Phc 13	2	111	117								0.541	0.456
Phc 25	9	121	123	127	129	131	133	139	143	145	0.595	0.683
										Average	0.599	0.607

Probability of Identity for the preliminary database:

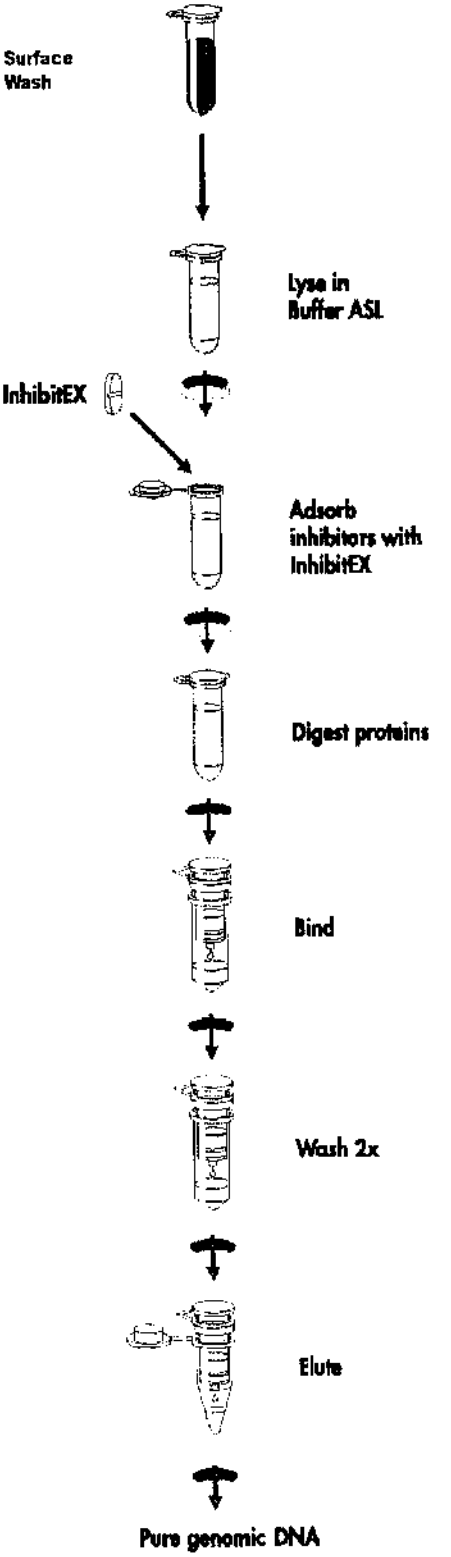
$PI = 1.41 \times 10^{-3}$

$PI_{sibs} = 1.57 \times 10^{-2}$

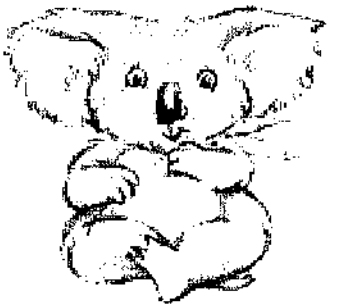
Appendix 5

Figure A3: Flow diagram of koala faecal DNA extraction method.

Modified from the QIAamp® DNA stool mini kit handbook, www.qiagen.com.



Appendix 6



A manual for collecting and transporting koala faecal pellets for molecular studies.

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March 22, 2003

Contents

1. Introduction

The application of molecular techniques to the management of endangered animals has become an invaluable tool to the conservation biologist (Avise 1994). Blood and tissue samples (liver, heart, kidney, spleen, muscle and brain) are the preferred biomaterial for genetic studies. However, acquiring these samples is often difficult, unsafe and stressful for both the animal and handler (Constable *et al.*, 1995; Kohn *et al.*, 1995; Kohn and Wayne, 1997; Levy, 1999).

In recent years, technological advances have made it possible to extract DNA from a variety of sources, including hair and faeces. Faeces collection is potentially the most reliable source of obtaining non- invasive DNA samples (Gerloff *et al.*, 1995; Constable *et al.*, 1995, Wasser *et al.*, 1997) because direct observation of the animal providing the sample need not be made and the collection and transport of faeces does not require approval from an animal ethics committee. This allows extensive sampling of a single animal or a group of animals within a population without disrupting the animals' normal behaviour (Gerloff *et al.*, 1995; Waits and Leberg, 2000).

Koala faecal pellets are easily distinguished from pellets of other animals in their homerange, by their shape and size (see figure 2.1). Faecal pellets are already used to indicate koala presence (Allen, 2000) and pellet distribution studies can be used in conjunction with radiotracking to investigate diet selection and habitat use (Ellis *et al.*, 1998; Phillips and Callaghan, 2000; Sluiter *et al.*, 2002).

However, it must be remembered that DNA recovered from faecal samples is of lower quality and quantity than DNA extracted from tissue (Constable *et al.*, 1995; Kohn and Wayne, 1997), and further complications, including DNA contamination, PCR inhibition and allelic dropout, are likely to be encountered (Kohn and Wayne, 1997; Taberlet and Luikart, 1999; Taberlet *et al.*, 1999). Therefore, it is imperative that all samples are collected, stored and transported properly to ensure that suitable biomaterials will be available for genetic and diagnostic testing.

2. Collecting koala faecal pellets

2.1 Appearance of the pellets

Although koala pellets are reasonably easy to identify, natural differences in the size and shape of pellets can occasionally make the identification and collection of koala pellets difficult. Approximately 9% of a koala's daily faecal pellet production will be found within 1m of the occupied tree (Ellis *et al.*, 1998). Therefore, e.g. if 174 pellets are produced per koala daily, up to 15 fresh pellets might be sampled at one location. The collection of pellets is also dependent on their physical condition; i.e. age and exposure to weather and the collectors experience in identifying a complete patina (shiny surface layer, see Figure 1), as these factors govern successful DNA extractions (Flagstadd *et al.*, 1999).

Although a complete patina is advantageous, the physical appearance of the pellets should not be used as a definitive guide to the presence of DNA, as pellets which have dried quickly in warm, dry climates are often better preserved than pellets from wet environments, which may appear fresh but can degrade quickly (Curtin *et al.* 2002; Ward 2002). Pellets can be classified in the following manner (Curtin *et al.*, 2002):

- **Fresh** (< 1 week old)- brown pellets covered in a shiny layer of mucus (patina), smelling strongly of eucalyptus;
- **Recent**- pellets shrunken, dried and not smelling strongly of eucalyptus, patina still present;
- **Old**- no visible patina, damaged but still identifiable as P. cinereus.

If a pellet is damaged or broken, i.e. classified as old, they should not be collected, but a record of the number of pellets in that location should be recorded. Pellet collection should be conducted from midday to midafternoon, to allow early morning dew to evaporate and allow fresh or wet pellets a chance to dry. Pellets should never be collected after fire or heavy rain, as there is little chance of DNA surviving these conditions. The ideal appearance of pellets which possess viable DNA is shown in Figure 1.

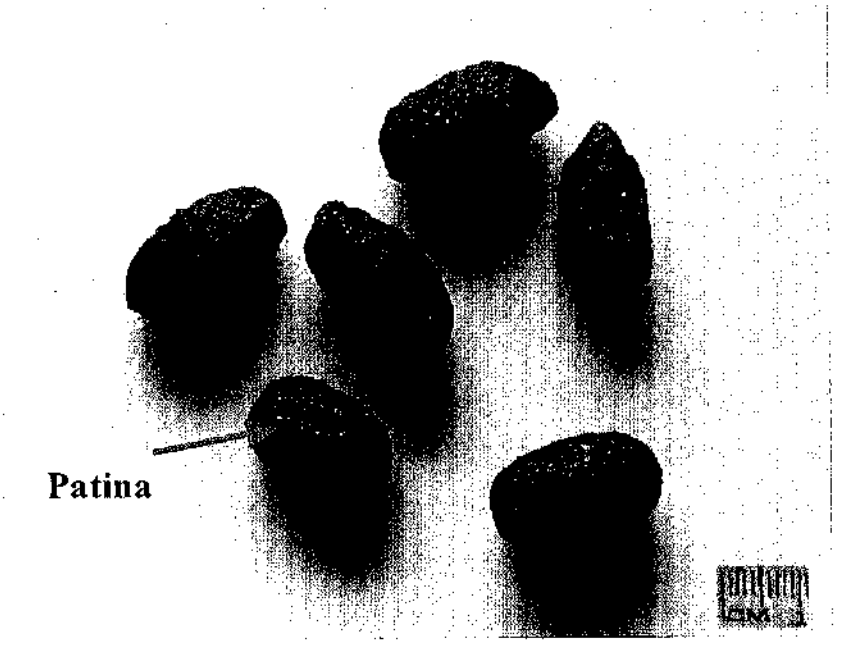

Figure 1: Appearance of koala faecal pellets collected for DNA extraction. Note the complete patina on all the pellets.

2.2 Collecting the pellets

2.2.1 Equipment required for collecting faecal pellets

Care should be taken when collecting faecal samples to ensure that contamination is minimised. It is recommended that the collector wear laboratory gloves at all times during the collection and use new, clean containers for the storage of each faecal sample collected at each location. Pellets should be collected into paper envelopes/bags or small plastic snap lock bags or into sealed containers e.g. plastic sample jars or empty film canisters.

Collectors should also carry a small spray bottle containing 70% ethanol. The spray nozzle should be no more than 2mm in diameter.

2.2.2 Collecting dry pellets

All pellets should be sprayed with 70% ethanol to lightly coat the pellets and the ethanol allowed to evaporate. The pellets should be sprayed four times in the north, south, east and west positions and the spray bottle held at least 30 cm from the pellets. This will preserve the pellets and prevent DNA degradation. All pellets may then be individually collected to avoid DNA transfer, in a suitably labeled container (see section 3).

2.2.3 Collecting wet pellets

If pellets are collected when wet or freshly deposited then individual pellets should first be sprayed with 70% ethanol to promote the preservation of the DNA and then collected individually by wrapping them in a small amount of absorbent paper (5 x 5 cm minimum size). The ideal types of paper to use include tissues, toilet paper, filter paper (Whatman 1mm), paper towel or cigarette paper.

Wet pellets from the same location should be individually collected in separate containers in the field and must be dried before transporting to the laboratory, in one of the following ways:

- Air dried in a warm dry area, for at least 24 hours or
- Oven dried at 30°C for 1hour.

3. Sample labelling

All faecal samples must have the following standard set of information accompanying them:

Date and time of collection
Field number
Scientific and/or common name of species collected
Locality
Number of pellets collected
Age of samples (new or old) and condition (wet or dry)

Permanent ink markers should be used for labelling and labels should be written directly on the sample bag or container. Alternatively a rough label should be placed inside the container, to ensure the collection data remains with appropriate samples. Field samples from a specific collection period should be accompanied with a datasheet describing the collection location, date, and descriptive notes on the sample, and any other information associated with the specimen. The condition of the animal dropping the pellets (if observed) should also be noted.

4 Transportation and long term storage of faecal samples

4.1 Transporting faecal samples

Transportation methods must aim to retain the samples as intact as possible during transit. Permission to transport faecal samples is not required from animal ethics committees. Therefore samples can be shipped in bulk with few problems.

Sturdy, internally padded packaging should be used when shipping pellets, this particularly applies to pellets collected in plastic or paper sample bags, which offer little to no protection during transport. All external labels should be clearly marked with permanent ink. A list of materials included, with relevant names, addresses and telephone numbers should be enclosed for the benefit of customs officials and to help recipients determine if the package was tampered with.

Before shipping, ensure that holidays (civic and religious) will not impede or delay the delivery of packages. It is also prudent to avoid shipping over the weekend.

4.2 Long term storage of faecal samples

All collection data should be archived in a computerised database or manual filing system. Faecal pellets should be stored in a dark place at room temperature or at –70 °C. A logbook should be kept with all samples identifying the sample number, date of storage and whether the samples have been removed for analysis.

5. Cost analysis for processing faecal samples

Laboratories should supply most PCR reagents (not including *Taq* polymerase), aerosol resistant pipette tips and buffers. Other reagents will depend upon the type of sequencing system utilised by the laboratory for analysing the DNA. The researcher is responsible for the cost of consumables such as polyacrylamide gels, QIAamp stool kits for carrying out DNA extraction, *Taq* polymerase and the radioisotope (if necessary).

The minimum time for processing 50 samples is 4 weeks (8h per day, 5 days a week). The cost of analysis begins at $180 a day (22.48 per hour) and varies depending upon the qualifications of the worker and day of the week. Any work conducted on a weekend or overtime will incur additional charges as follows:

Over time Monday – Saturday, first three hours 150% - $28.10 per hour

Over time Monday – Saturday, >3 hours and Sundays 200% - $37.47 per hour

Public Holidays, 250% - $46.83 per hour.

An example of the costs involved for processing 50 faecal samples in a laboratory using autoradiography:

Items supplied by the laboratory	Cost including delivery and GST ($)
dNTPs (100 mM stock slns)	240
10F pipette tips (10pack, 90 per box)	159.50
TBE buffer (10 pack)	90.20
Mirosatellite/ mtPCR primers	500
Developer and fixer solutions	40
Cellophane membrane backing sheets	172.40
Whatman 3MM chromatography paper	421.30
Ammonium Persulfate	60.50
PCR tubes and caps	447.70*
Total	2133.20
35% of total	746.62

A maximum 35% charge of these costs would be sufficient to replace these items as necessary.

* May vary depending upon the type of tubes used, i.e. 0.2 mL or 0.3 mL.

Cost consumables for 50 samples:

Consumables	Cost including delivery and GST ($)
2 x Stool kits	668.80
Sequagel- 6	165
dATP (500 μCi)	613.80*
BioTAQ	295.35
Hyperfilm	217.80
subtotal	1960.75
+10% error surcharge	196
+35% of our costs	746.62
Total	2903.37

*- if autoradiographs are used in the laboratory.

Minimum cost of analysis (8hr per day five days per week):

180 x 20 = $3596.80

Minimum cost for analysing 50 samples (no weekend work or overtime) = $ 6500.17

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