Strength in diversity: Investigating methods to determine genetic variability in a captive colony of small carnivorous marsupials, the fat-tailed dunnart *Sminthopsis crassicaudata*

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ABSTRACT

The importance of preserving genetic diversity is emerging as a critical issue in conservation. Loss of genetic diversity has serious implications for the fitness and viability of species, populations, ecosystems, and ecological processes. Advances in molecular monitoring tools and techniques are increasing their scope and application to a range of conservation management techniques. The aim of this project is to investigate methodologies for the examination of genetic variation within a captive colony of fat-tailed dunnarts (*Sminthopsis crassicaudata*), a small carnivorous marsupial of the order Dasyuromorphia. DNA was extracted from 100 preserved specimens from the captive colony and three preserved wild specimens. Polymerase chain reaction (PCR) was used to amplify cytochrome *c* oxidase (COX), ω-globin, and D-Loop Control Region (*CR*) genes. DNA sequencing was conducted on the COX gene for 14 individuals to examine levels of variation at that locus. A draft fat-tailed dunnart genome was then examined *in silico* to identify perfect tri-nucleotide microsatellites and primers. Primers for 20 prospective microsatellite loci were synthesized and screened against a subset of the population. The DNA sequencing revealed no variation within the samples from the captive colony, and a high degree of homology (99%) to other fat-tailed dunnart genomes. The results of the microsatellite design and amplification successfully isolated a single microsatellite which was found to contain both polymorphism and heterozygosity. To our knowledge, this is the first time a microsatellite has been successfully isolated within the fat-tailed dunnart genome. The success of the methods trialed in this project will allow for the development of a suite of microsatellites within the fat-tailed dunnart genome that can be applied to population genetic studies of fat-tailed dunnarts and other marsupial species.

KEYWORDS

Fat-tailed dunnart, *Sminthopsis crassicaudata*, captive breeding, founders, genetic variation, genetic diversity, phylogenetics, microsatellites.

ABBREVIATIONS AND GLOSSARY

Allele: a variation of a gene

COI: Cytochrome *c* oxidase I

COX: Cytochrome *c* oxidase

ESU: Evolutionarily Significant Unit

HVWC: Hidden Vale Wildlife Centre

IUCN: International Union for the Conservation of Nature

Linkage disequilibrium: The non-random association of alleles at two or more loci in a given population.

mtDNA: Mitochondrial DNA

MU: Management Unit

PCR: Polymerase chain reaction

Phylogenetics: The study of evolutionary relationships among biological entities, often species, individuals, or genes.

qPCR: Quantitative polymerase chain reaction

Reciprocal monophyly: In the analysis of gene tree data between sister species, the situation where haplotypes are more closely related within the species than between the two sister species.

RFLP: Restriction fragment length polymorphism

SNP: Single nucleotide polymorphism

SSR: Single strand repeat

TBE: Tris-borate-EDTA buffer

UTR: Untranslated region

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1 INTRODUCTION

The truth of the statement “a thousand-mile journey begins with a single step” is comprehensively and beautifully illustrated by evolution at the genetic level. A change in a single base in a single DNA molecule may spark a cascade of changes firstly in an individual organism and ultimately a species (Abdul-Muneer 2014). Genetic diversity is vital to the general resilience of a population in terms of that population’s ability to adapt to environmental change, withstand stochastic events and avoid the deleterious effects of inbreeding depression and increased allelic homogeneity (Frankham 1995; Crnokrak & Roff 1999; Woodworth et al. 2002; Koepfli & Gooley 2020). Population genetic health has a cascading effect that ultimately impacts species and habitat diversity, and stability of ecological processes (Mimura et al. 2017).

The actual or threatened loss of genetic diversity is of increasing concern. Anthropogenic factors are exerting unprecedented stress on the earth’s natural systems. Chief among these are habitat fragmentation and destruction, climate change, poaching, and illegal trafficking (Koepfli & Gooley 2020). It is now accepted that the world is experiencing what is known as the sixth mass extinction event: the erosion of the richest biodiversity ever to exist on the planet (Ceballos et al. 2017). Even more serious than species extinctions, the usual metrics by which the severity of this extinction event is measured, is the phenomenon of population extinction and range contraction (Ceballos et al. 2017). Population extinction is necessarily a prelude to species extinction and is likely to have negative cascading consequences far into the future in terms of degradation of ecosystems and loss of genetic diversity (Ceballos et al. 2017).

Australia has an extremely poor track record when it comes to the decline and loss of endemic species. Our fauna is among the most unique in the world, comprising ancient monotreme lineages, diverse marsupial species, and a range of endemic eutherians (Woinarski et al. 2015). In the two centuries following European colonisation, however, Australia's native mammal fauna has suffered, and continues to suffer, rapid decline (Woinarski et al. 2015). This is despite the fact that our position as a nation of relatively low population density, high economic wealth and large remaining areas of natural environments means we are not as subject to the factors that, worldwide, are largely responsible for biodiversity decline (Woinarski et al. 2015).

Other than those species that attract high public sympathy, the fate of most Australian mammals goes largely unnoticed. Population monitoring is patchy at best and little is known about the true status of many species (Woinarski et al. 2014). Native eutherian mammals, the bats and rodents, for example, make up nearly half of Australia's terrestrial fauna, yet receive little research attention (Fleming & Bateman 2016). Little is known about the true conservation status of many of these species (Fleming & Bateman 2016). Other species, such as the platypus (*Ornithorhynchus anatinus*) or the echidna (*Tachyglossus aculeatus*) have received more attention for their physiology or taxonomy than for population trends or abundance (Fleming & Bateman 2016).

Australia's pattern of mammalian extinction and loss began with the extinction of the megafauna between 60,000 and 20,000 years ago (Woinarski et al. 2014). This coincided with the arrivals of the first humans in Australia and periods of rapid climate change (Woinarski et al. 2014). The trend was redoubled with the advent of European colonisation in 1788 (Woinarski et al. 2014), famously resulting in the loss of the thylacine (*Thylacinus*

cyanocephalus) in the early part of the 20th century. The ongoing rapid decline of Australia's mammalian fauna far exceeds that of other continents, or of other taxonomic groups within Australia (Woinarski et al. 2014).

European colonisation heralded significant changes in land use and the introduction of many non-native species. Since that time, at least 28 endemic land mammal species have become extinct (Woinarski et al. 2015). This figure represents 11% of the total 273 endemic Australian land mammal species and 35% of the modern world's mammal extinctions (Woinarski et al. 2015). A further 21% of Australia's land mammals are listed as Threatened and 15% as Near Threatened (Woinarski et al. 2015). Moreover, much of the loss of Australia's endemic land mammals has been of species that are phylogenetically distinct, representing a disproportionate loss of the planet's genetic diversity (Woinarski et al. 2015).

The importance of understanding and preserving genetic diversity is receiving increasing worldwide attention. At the 2010 Convention on Biological Diversity, the preservation of genetic diversity constituted Aichi Biodiversity Target 13 (Hoban et al. 2013; Convention on Biological Diversity 2020; Forcina & Leonard 2020). Maintenance of genetic diversity is further recognised by the International Union for the Conservation of Nature (IUCN) as one of the three levels: genetics; species; and ecosystem where biodiversity must be preserved (Frankham 1995; England et al. 2003). Although increasingly enshrined in law, the reality of incorporating these principles into the management of free-living and captive populations is often poorly understood and not a feature of conservation plans (Forcina & Leonard 2020).

Advances in genetic sequencing and molecular marker technologies are now making it easier to incorporate the preservation of genetic diversity into broader conservation plans. Model species such as the fat-tailed dunnart (*Sminthopsis crassicaudata*) can be used to facilitate

these processes. Fat-tailed dunnarts make an ideal genetic, biological, and reproductive conservation model as they are easy to keep and breed in captivity and multiple generations can be bred relatively quickly (Bennett et al. 1990; Morton 1991; Noy et al. 2017). The information gleaned from studies on species such as the fat-tailed dunnart can then be applied to other marsupial species.

1.1 FAT-TAILED DUNNARTS (*SMINTHOPSIS CRASSICAUDATA*)

Fat-tailed dunnarts are small, nocturnal carnivores, feeding primarily on insects and other invertebrates (Morton 1991). Males and females both weigh approximately 16 g at maturity, with a head and body length of 90 mm and a tail length of 60 mm (Bennett et al. 1990). Males reach sexual maturity at around 7-8 months of age and females at around three months (Bennett et al. 1990). Females can produce three litters of up to 10 pouch young per year, with breeding occurring from July to February (Bennett et al. 1990; Noy et al. 2017).

Fat-tailed dunnarts are one of the most common and wide ranging of the *Sminthopsis* genus (Cooper et al. 2000). The species' status is listed as Least Concern in the IUCN Red List of Threatened Species (Burbidge et al. 2016) and unlike many other dasyurids it has likely benefitted from ongoing post-colonisation land clearing activities. This is because of its preference for open grasslands and shrublands that has allowed it to expand its range into land cleared for cropping and grazing activities (Morton 1991).

The fat-tailed dunnart occurs within a wide range of habitats. This includes areas of open woodland, low shrubland, tussock grassland and farmlands (Morton 1991). The average annual rainfall ranges from 550 mm in the southern and eastern parts of its distribution to less than 150 mm in the arid centre of Australia (Morton 1978). There are two recognised

taxonomic subspecies: *S. crassicaudata crassicaudata* whose range includes the less arid regions of Western Australia, South Australia, New South Wales and Queensland, and *S. crassicaudata centralis* which is found in arid central Australia (Cooper et al. 2000). There are various morphological differences within and between the two subspecies, including coat colour, foot-pad configuration, and ear and tail length, with a marked north-south geographic cline (Morton 1978; Cooper et al. 2000). Generally, *S. c. centralis* is characterised by a sandier coat colour, longer ears and tail, and granular foot-pads. However, the aforementioned geographic cline in morphological features makes it difficult to accurately distinguish between the two subspecies based on physical characteristics alone (Morton 1978; Cooper et al. 2000).

Genetic analyses of the fat-tailed dunnart metapopulation shows evidence of a species subdivision, however the distribution does not match that of the taxonomic subspecies (Fig. 1) (Cooper et al. 2000). Examination of allelic variation in blood proteins and genetic variation of the mitochondrial Control Region (*CR*) has revealed two distinct clades: the southeast clade, comprising populations from southeast South Australia and Victoria; and the northwest clade, comprising all remaining populations (Cooper et al. 2000). Earlier studies provided similar findings, with the Murray river proposed as a likely geographic barrier between the two populations (Fig. 1) (Hope et al. 1986).

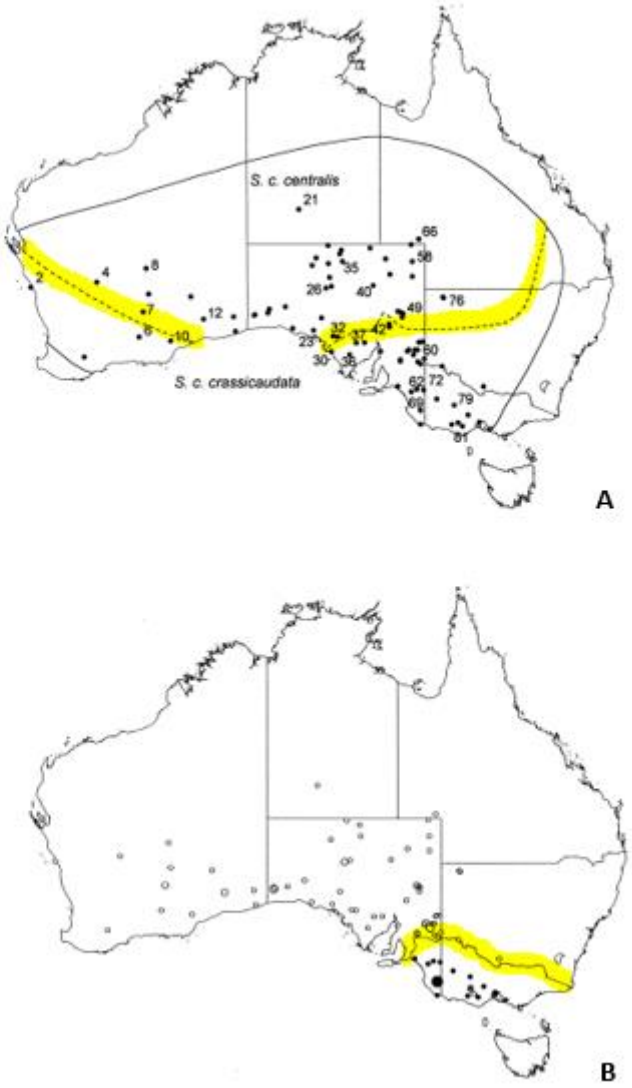


Figure 1: Comparison of the distribution of the two taxonomically described subspecies of *S. crassicaudata* with that of the genetically distinct clades. Image A shows the distribution of *S. c. centralis* and *S. c. crassicaudata* respectively. The subspecies boundary is marked by the highlighted dotted line. Image B shows the distribution of the genetically distinct southeast (solid dots) and northwest (hollow dots) clades. The Murray river (highlighted) has been proposed as a likely geographic barrier between the two clades (Cooper et al. 2000).

Hybridisation has been observed between the northwest and southeast clade, although the relative fertility of hybrids compared to non-hybrids was not known at the time Cooper et al. (2000) undertook their study and does not appear to have been documented since. The results from Cooper et al. (2000) provide evidence of reciprocal monophyly, indicating that the degree of genetic relatedness within the northwest and southeast clades is substantially greater than the degree of relatedness between the two clades.

The two fat-tailed dunnart clades, therefore, demonstrate sufficient variation to be classed as Evolutionarily Significant Units (ESUs) (Cooper et al. 2000). ESUs are historically isolated populations with distinct evolutionary potential that are reciprocally monophyletic for mtDNA alleles (Moritz 1994). Mitochondrial alleles, inherited through the maternal line, have a proportionally higher likelihood of being passed on to subsequent generations than bi-parentally inherited nuclear alleles, only half of which are transmitted from each parent. They therefore have a lower effective population size than nuclear alleles which are more likely to be diluted in a population due to the genetic contribution of the males. Non-coding regions of the mitochondrial genome such as the Control Region evolve rapidly, resulting in high relative substitution rates and sequence variation. Mitochondrial DNA, therefore, will demonstrate reciprocal monophyly sooner than nuclear alleles. ESUs, however, also show significant divergence of allele frequency at nuclear loci (Moritz 1994).

2 LITERATURE REVIEW

2.1 THE IMPORTANCE OF GENETIC DIVERSITY

The importance of genetic variability to the health and functioning of individuals, populations and ecosystems is undisputed. The increasing attention being given to genetic variability by

international authorities such as the IUCN and the Convention on Biological Diversity speaks to the critical importance of this issue as evidenced by earlier research. Among the other threats currently faced by the world's wildlife, loss of genetic variability must be regarded as one of the most serious in the light of its long-term detrimental effects on population resilience, and the fact that these effects may not become apparent for many generations (Ceballos et al. 2017).

The term 'genetic diversity' here refers to intraspecific variation in genetic composition and expression (Mimura et al. 2017). It comprises variation in how genes are expressed as well as 'functional' and 'neutral' variation in genetic sequences (sequence variation). Functional sequence variation is the expression of morphological, physiological, and other functional traits such as physical appearance, body composition and disease resistance (Mimura et al. 2017). These are factors that will, ultimately, determine an organism's fitness and survivability in terms of its reproductive success and how well it is adapted to its environment (Mimura et al. 2017). Functional variation has important flow on effects for population viability as fitness parameters such as survival and reproduction can be heavily negatively impacted by reduced genetic variability (Mimura et al. 2017).

Neutral sequence variation refers to variation within sections of the genome that do not code for functional traits (Mimura et al. 2017). Such variation is not expressed morphologically or physiologically, and therefore does not have the kind of direct impact that loss of functional variation may have (Mimura et al. 2017). Neutral sequence variation does, however, serve as an important index for determining parameters such as effective population size, gene flow, evolutionary potential, and genetic integrity (Mimura et al. 2017). Among the most useful molecular markers within the non-coding region of the genome are microsatellites (Vieira et

al. 2016). These repetitive, non-coding regions serve as highly informative indicators of parameters such as estimation of gene flow, diversity and infraspecific relatedness due to their evolutionary instability and high mutation rates (Vieira et al. 2016).

Functional and neutral sequence variation has implications for the health and functioning of populations, communities, and ecosystems. The phenotypic changes, such as changes in physical appearance or physiological function that we associate with changes in functional genetic sequences can also be impacted by changes in neutral or non-coding sequence variation (Mimura et al. 2017). For example, variations in microsatellites in sections of the genome known as untranslated regions (UTRs) may affect transcription and translation of the genetic code as well as modulation of gene expression (Vieira et al. 2016; Bagshaw 2017).

In conservation, much of the focus still centres on ecosystems, communities and interspecific interactions when attempting to manage the effects of anthropogenic environmental change, rather than on intraspecific variation and genetic diversity (Mimura et al. 2017). However genetic variation is of critical importance (Mimura et al. 2017). At the population level, genetic variation increases stability by allowing for greater species resilience, especially in response to environmental change; reduces the negative effects of inbreeding depression; and increases effective population size (Mimura et al. 2017). At the level of communities or ecosystems, genetic variation results in increased species and habitat diversity and greater stability of ecological processes such as pollination, decomposition, and nutrient cycling (Hoban et al. 2013; Mimura et al. 2017).

Most populations of conservation significance have had their genetic diversity impacted at some level, whether through reduction in numbers or through reduced gene flow due to increased habitat and population fragmentation or isolation (Forcina & Leonard 2020).

Increasing fragmentation and reduction of habitat area means the effects of increased isolation and potential loss of genetic variability have the potential to impact almost any species regardless of its conservation status (Koepfli & Gooley 2020).

Reduced gene flow and increased gene flow can both have negative implications for a population's genetic variability and health (Frankham 1995; Woodworth et al. 2002; Mimura et al. 2017). The potential negative effects of reduced gene flow may include inbreeding and inbreeding depression, accumulation of deleterious mutations, founder effects and genetic adaptation to captivity leading to reduced wild fitness (Frankham 1995; Woodworth et al. 2002; Charlesworth & Willis 2009; Kennedy et al. 2014; Mimura et al. 2017). Those of increased gene flow may include outbreeding effects such as hybridisation, reduced speciation, and the introduction of maladapted genes (Frankham 1995; Woodworth et al. 2002; Rice & McQuillan 2018).

Reduced gene flow

Inbreeding refers to the mating of closely or moderately-closely related individuals, for example parent-child or aunt-nephew, resulting in increased homozygosity in the offspring (Marshall et al. 2002; Charlesworth & Willis 2009). Inbreeding depression is a negative outcome of this increased homozygosity, the potentially harmful impacts of which have been documented since at least the time of Charles Darwin (Charlesworth & Willis 2009). Left unchecked, inbreeding depression can ultimately lead to extinction events on either a local population or species-wide scale (Charlesworth & Willis 2009).

Inbreeding depression manifests as a reduction in the expression of fitness-related characteristics of fertility, survival, and growth, as well as the increased likelihood of genetic abnormalities or disease (Marshall et al. 2002; Charlesworth & Willis 2009). Inbreeding

depression is usually measured through fitness-related traits such as juvenile survival or number of eggs laid; or metric traits such as ejaculate volume or plant height (Crnokrak & Roff 1999). The genetic causes of inbreeding depression include the expression of deleterious mutant alleles and the loss of heterozygote advantage (Charlesworth & Willis 2009; Singh & Kulathinal 2017). Furthermore, inbreeding depression is cumulative, resulting from the combined expression of deleterious mutations at different loci. The effects tend to accumulate over generations, although fitness measures can be halved in as little as a single generation of inbreeding (Charlesworth & Willis 2009).

Deleterious mutations may naturally be present in any given population, but are generally recessive and so masked in the heterozygote form (Charlesworth & Willis 2009). These mutations may accumulate in a population as a result of inbreeding or genetic drift, and the increased number of homozygotes in an inbred population leads to an increased frequency in the expression of these alleles and consequent reduction in fitness (Charlesworth & Willis 2009; Kennedy et al. 2014). The increased homozygosity that results from inbreeding may also lead to a loss of heterozygote advantage, wherein the heterozygote expression of a particular combination of alleles conveys greater fitness that either the homozygous dominant or homozygous recessive combination (Charlesworth & Willis 2009; Singh & Kulathinal 2017).

The general assumption that inbreeding is always detrimental and that organisms will consequently always evolve inbreeding avoidance behaviours has been challenged on several fronts (Waser et al. 1986; Frankham 1995; Szulkin et al. 2013). In wild populations, due to the cumulative effects of finite population size, some degree of inbreeding will occur naturally over hundreds of generations (Frankham 1995). Inbreeding is not without benefits, such as

the ‘inclusive fitness’ benefit of inheriting the same allele from both parents (Szulkin et al. 2013). The inclusive fitness benefit derives from the increased likelihood of a given allele being passed to an individual through both parental lines, thus increasing the likelihood of that allele’s ongoing survival (Szulkin et al. 2013). Certain mating systems may also benefit from inbreeding behaviours, such as in the case of lek-forming species, for example the buff-breasted sandpiper (*Tryngites subruficollis*) or the black grouse (*Lyrurus tetrix*), where the dominant or competitively successful male has the primary choice of females (Foster 1983; Waser et al. 1986). Under such circumstances, inbreeding may be a beneficial choice to subordinate males, on the grounds that any chance to pass on one’s genes is better than no chance at all (Waser et al. 1986). The cost of dispersal, including the increased risk of predation and the need to establish a home range or territory, must also be offset against the cost of inbreeding (Waser et al. 1986).

There is, however, sufficient empirical evidence to show that, over time, inbreeding will reduce the reproductive fitness and survival of a population (Frankham 1995; Frankham 1998; Crnokrak & Roff 1999; Woodworth et al. 2002; Koepfli & Gooley 2020). Furthermore, this effect is exacerbated where ecosystems are consistently subject to the compounding effects of anthropogenic disruption and stochastic events (Koepfli & Gooley 2020).

Increased gene flow

Hybridisation, the production of offspring from the mating of two different species or varieties of organism, is a natural process of speciation that leads to the emergence of novel genetic combinations (Abbott et al. 2013; Rice & McQuillan 2018). Such novel combinations can have varying impacts on phenotypes and may be advantageous or disadvantageous (Abbott et al. 2013; Rice & McQuillan 2018). In the case of domesticated or inbred species, hybrid offspring can demonstrate greater heterozygosity and consequently greater genetic fitness than their parents, a phenomenon known as hybrid vigour (Charlesworth & Willis 2009; Rice & McQuillan 2018). In free-living populations, hybridisation may facilitate or retard the process of speciation (Abbott et al. 2013; Rice & McQuillan 2018). For example, some common domestic crosses, such as mules (*Equus mulus*), may demonstrate higher learning ability than their parental species of horse (*Equus caballus*) and donkey (*Equus asinus*) (Rice & McQuillan 2018). Conversely, a study of food-cache recall ability in hybrid offspring of black-capped chickadees (*Poecile atriacapillus*) and Carolina chickadees (*P. carolinensis*) showed that hybrid offspring had poorer memory recall than the non-hybrid parent species (Rice & McQuillan 2018).

While increased gene flow may increase speciation through processes such as hybridisation, it can also have the reverse effect and reduce speciation. This can be particularly problematic in captive breeding and translocation programs where maladapted genes are introduced or locally adapted alleles are lost (Mimura et al. 2017; Gallardo-Alvárez et al. 2019). Reduced speciation can lead to the loss of adaptations that are specific to local conditions such as disease resistance or heat tolerance (Mirkena et al. 2010).

Maintaining genetic diversity

The genetic diversity of the founder individuals of any population is of critical importance (Gustafson et al. 2018; Gallardo-Alvárez et al. 2019). Founder genetic diversity will ultimately impact on factors such as potential for inbreeding or outbreeding depression, loss of allelic diversity due to genetic drift, and fixation of deleterious alleles (Gustafson et al. 2018; Gallardo-Alvárez et al. 2019). This is a common and well-documented problem among domestic animals where breeding for desired morphological traits leads to inbreeding depression and an increase in genetic deformities and disease (Gustafson et al. 2018; Yordy et al. 2019). Founder genetic diversity is also a consideration when managing free-living or captive populations, whether in zoo environments, larger free-ranging environments such as managed reserves, or in their natural habitat (Woodworth et al. 2002; England et al. 2003; Mulvena et al. 2020). It is also necessary to know the geographic origins of the founders as this could cause problems when introducing new animals or pairings, for example through the introduction of maladapted genes (Mimura et al. 2017; Gallardo-Alvárez et al. 2019).

In terms of fitness for release, captive populations are susceptible to deterioration due to the effects of any or all of: inbreeding depression; loss of genetic diversity; genetic adaptation to captivity; and the accumulation of new mutations (Woodworth et al. 2002). A study of the model species the vinegar fly *Drosophila melanogaster*, for example, showed that the accumulation of rare, largely recessive alleles appears to be a major contributing factor to genetic adaptation to captivity (Woodworth et al. 2002). Ensuring genetic fitness, therefore, is an important part of captive animal management and many captive populations are managed with the short- or long-term goal of ultimately returning these populations to the wild (Woodworth et al. 2002).

Strategies to facilitate gene flow, however, can help to reverse the effects of genetic erosion and inbreeding depression of captive populations (Alpers et al. 2016). First, introduction of immigrants has the capacity to effectively improve connectivity between fragmented populations, restore population size and increase genetic diversity (Gallardo-Alvárez et al. 2019). Second, equalisation of family size allows more individuals breeding opportunities particularly, for example, among polygynous species such as the eastern grey kangaroo (*Macropus giganteus*) where fewer males might otherwise be granted the opportunity to mate (Waser et al. 1986; Woodworth et al. 2002). Third, fragmentation of captive populations in order to isolate gene pools may also be effective as this has been shown to minimise selection for undesired traits, although care needs to be taken to avoid inbreeding depression (Woodworth et al. 2002). Finally, the use of captive environments that mimic wild conditions as nearly as possible may also be effective in minimising genetic adaptation to captivity (Woodworth et al. 2002).

The potential negative effects of inbreeding depression are a factor in the management of both captive and free-living populations. The incidence of inbreeding depression in captive populations may be biased up as a result of poor husbandry or breeding techniques (Crnokrak & Roff 1999). On the other hand, reduced effective population size and barriers to inter-population gene flow may increase the risk of genetic erosion and inbreeding depression in free-living populations (Alpers et al. 2016). Where free-living populations are inbred they generally exhibit statistically significant levels of inbreeding depression (Crnokrak & Roff 1999).

The negative impacts of inbreeding depression have been shown to be higher in free-living mammal populations than captive populations. This may be a consequence of factors such as

the better care available to captive animals as opposed to the naturally harsher conditions of wild environments including: fluctuations in climatic conditions; unpredictability of food availability; intra and interspecific competition; and stochastic events such as fire (Frankham 1995; Crnokrak & Roff 1999; Woodworth et al. 2002). The characteristically low heterozygosity and higher numbers of lethal equivalent alleles present in inbred populations means they may be less fit to survive such events (Crnokrak & Roff 1999). Studies on *D. melanogaster*, for example, have demonstrated that captive populations of this model species show significant deterioration in genetic fitness on return to the wild. (Woodworth et al. 2002). In larger *D. melanogaster* populations this deterioration is primarily due to genetic adaptation to captivity, in smaller populations, inbreeding depression (Woodworth et al. 2002).

2.2 MOLECULAR ANALYSES

A thorough understanding of genetics is an essential component of species management and conservation. This is particularly important when the conservation goals for the species are to mitigate the effects of reduced genetic variation through the introduction of new breeding stock. To be effective, the conservation manager must understand the natural historical processes and recent anthropogenic influences that have shaped a species' genetic structure (Benfer et al. 2014). For example, the population may have undergone divergence and adaptation to local conditions leading to outbreeding depression or the loss of unique lineages (Benfer et al. 2014).

As the power and accuracy of molecular monitoring tools and techniques increases, so too does the scope of their application to conservation strategies. Genetic markers are used to gather information about allelic variation (Schlötterer 2004), and a wide range of markers are

available, with application to a variety of management techniques. These include: identification of conservation management units such as Evolutionarily Significant Units (ESUs) or Management Units (MUs) (Moritz 1994; Cooper et al. 2000; Hoban et al. 2013); species identification; population monitoring; determining parentage and pedigree information; and monitoring adaptation to different or changing environments (Schlötterer 2004; Hoban et al. 2013).

Numerous molecular techniques have been developed over the past 50 years. Among the molecular tools available are allozymes, restriction fragment length polymorphisms (RFLPs), microsatellites and single nucleotide polymorphisms (SNPs) (Schlötterer 2004), and more recently, high throughput genome sequencing (Forcina & Leonard 2020). Each has its advantages and disadvantages depending on its application. Allozymes (*allo* = different, *zyme* = contraction of enzyme) were the first true molecular markers to be identified. They function by identifying underlying DNA sequence variation through observed changes in enzyme charge (Schlötterer 2004; Forcina & Leonard 2020). The differences in charge correspond to the substitution of charged amino acids and appear as different migration rates in an electric field. The substituted amino acids are conferred through changes in the DNA but are relatively crude because mutations leading to neutral or redundant substitutions remain invisible (Schlötterer 2004; Forcina & Leonard 2020). RFLPs were used to gauge DNA variation by detecting patterns caused by single base substitution in a restriction enzyme's recognition sequence (Schlötterer 2004). RFLPs thus allowed analysis of non-coding as well as coding sequences, however only some DNA changes were visible by this method (Schlötterer 2004). Microsatellites and SNPs are among the most popular and widely used tools, with SNPs gaining increasing favour with the evolution of next-generation sequencing techniques (Nielsen et al. 2020). In particular, when analysing a species for the first time, mitochondrial

DNA (mtDNA) sequences, which are inherited through the maternal line, function as an important and cost-effective target for analysing genetic diversity and structure (Koepfli & Gooley 2020)

The bulk of data in early phylogeographic analyses tended to be drawn from mtDNA (Avise et al. 2016). However because of its matrilineal inheritance, it can only provide us with a portion of the hereditary pedigree of a species (Avise et al. 2016). The nuclear DNA must also be examined in order to gain a complete picture (Avise et al. 2016). Next generation sequencing has increased the availability and ease with which this can be done (Avise et al. 2016).

Microsatellites, also known as Single Strand Repeats (SSRs) serve as excellent genetic markers because they are typically selectively neutral. Any mutations that occur are unlikely to be subject to the same degree of selection as coding regions of the genome, which generally leads to the formation of new alleles at a given locus (Vieira et al. 2016; Forcina & Leonard 2020). Microsatellites are non-coding sections of DNA consisting of unique tandem repetitive arrays of DNA sequences with repeat sizes of anywhere from one to six base pairs (bp), with two to four bp being usual and core repeats of three to five being preferred for parentage analyses (Abdul-Muneer 2014; Vieira et al. 2016). It is the differences in the numbers of repeats of the repeat unit, also known as the motif, that causes the observed variants, or polymorphisms, between individuals (Vieira et al. 2016). These repeat polymorphisms may occur due to a phenomenon known as strand-slippage replication, where the template and nascent DNA strands are mismatched during the process of replication, causing either contraction or expansion of the number of repeats (Vieira et al. 2016). Recombination errors such as unequal crossing over can also result in repeat length polymorphisms (Vieira et al. 2016). Any given SSR locus may therefore consist of different alleles (Vieira et al. 2016).

Microsatellites are found in high numbers within the genome of most eukaryotes (Schlötterer 2004; Vieira et al. 2016). They provide one of the most highly informative and useful types of genetic marker for analysing pedigree, population structure and genome variation, even among closely related populations (Schlötterer 2004; Abdul-Muneer 2014; Vieira et al. 2016). It is the co-dominant, multi-allelic nature of microsatellites, together with the fact that they are easily experimentally reproducible, transferable among related species and capable of isolating large numbers of loci that makes them so valuable as genetic markers (Schlötterer 2004; Abdul-Muneer 2014; Vieira et al. 2016).

There is wide support in the literature for the use of microsatellites and mtDNA in studies of marsupial genetics. Examples include: 1) an examination of gene flow at different time scales to assess the phylogeography of the southern hairy-nosed wombat (*Lasiorhinus latifrons*) (Alpers et al. 2016); 2) population monitoring and remote censusing of the highly endangered northern hairy-nosed wombat (*L. krefftii*) (Sloane et al. 2003); and 3) a study of social and genetic structure in the endangered sandhill dunnart (*Sminthopsis psammophila*) (McLean et al. 2014).

Within the mitochondrial genome, the following genes have been found to be informative for examining population structure and genome variation: the control region (*CR*) and cytochrome *c* oxidase (COX). *CR* is widely used in studies of species boundaries and population structure (Labrinidis et al. 1998; Cooper et al. 2000) as it is a rapidly evolving region of the genome that can be used to assess gene flow over both long- and short-term evolutionary time scales (Labrinidis et al. 1998).

Cytochrome *c* oxidase is well established in the literature as an effective tool for bioidentification of organisms at the levels of phyla, class, and species (Hebert et al. 2003;

Costa et al. 2007). This has been demonstrated across a diverse range of taxonomic groups, including the class Hexapoda, order Lepidoptera (Hebert et al. 2003) and sub-phylum Crustacea (Costa et al. 2007). The effectiveness of COX lies in the fact that there are robust universal primers available, meaning it can be used across most taxa (Hebert et al. 2003). Cytochrome *c* oxidase also has a higher range of phylogenetic signal than other mitochondrial genes (Hebert et al. 2003). It demonstrates a higher rate of molecular evolution than the other commonly targeted mitochondrial genes, 12*S* and 16*S* rDNA, allowing accurate detection of closely related species as well as intraspecific phylogeographic groups (Hebert et al. 2003). An example of the effectiveness of COX as a taxonomic tool was demonstrated in a study that tested the classification of lepidopterans (Hebert et al. 2003). Cytochrome *c* oxidase was 100% successful in correctly identifying and classifying the target organisms (Hebert et al. 2003).

Within the nuclear genome, ω-globin has recently begun to be employed in studies of phylogenetic relationships between congenerics, including the *Sminthopsis* genus (Blacket et al. 2006b). The ω-globin gene is widespread in marsupials and its evolution is thought to pre-date the separation of birds and mammals (Wheeler et al. 2001).

2.3 RESEARCH QUESTIONS

2.3.1 Project aims

This project aims to investigate genetic variation within a species using a captive fat-tailed dunnart colony as a model. The captive colony used is housed in the University of Queensland's Hidden Vale Wildlife Centre (HVWC) located in Grandchester, Queensland. This investigation will involve analyses of mtDNA and nuclear marker genes as well as the identification and amplification of microsatellite markers within the fat-tailed dunnart

genome, using a combination of *in silico* and *in vitro* analyses. To the best of our knowledge, microsatellites have not previously been isolated within the fat-tailed dunnart genome. This project, therefore, will open pathways to further research into population genetics in the fat-tailed dunnart and other marsupial species.

Specifically, this project will: 1) undertake polymerase chain reaction (PCR) amplification and sequence analysis of candidate mitochondrial and nuclear genome loci using existing established methods; and 2) identify novel nuclear microsatellite markers within the fat-tailed dunnart genome using a combination of *in silico* analyses and PCR amplification.

2.3.2 Project background and significance.

The fat-tailed dunnart colony at the HVWC was established in 2012 from an unrecorded number of founder animals sourced from three captive colonies in Melbourne, Adelaide and Newcastle, Australia (D. Adam 2020, pers. comm., 14 September). The colony currently consists of approximately 130 individuals, making it one of the largest extant captive colonies of fat-tailed dunnarts in Australia. Additionally, every deceased specimen from the colony has been preserved, creating a resource of approximately 1,600 specimens, and so the full genetic record of the colony is available (D. Adam 2020, pers. comm., 3 July).

The growing availability and sophistication of molecular tools for genetic analysis means that the ability to monitor and understand genetic variability is becoming increasingly accessible. The HVWC fat-tailed dunnart project is a step in this direction. The knowledge gained from this project will be used to inform decisions and directions of larger-scale projects, as well as open up research streams into relationships between captive breeding, genetic variability, and fitness for release into the wild. It will enable the development of models that will facilitate research into other marsupial species. Potential applications include populations

that have been established with founders of unknown genetic origin or relatedness, or where breeding programs have been undertaken with incomplete knowledge of genetic variability, existing levels of inbreeding, or degree of relatedness of breeding pairs.

Examples of at-risk species where such a model could be applied include the Julia Creek dunnart (*S. douglasi*) and the Tasmanian devil (*Sarcophilus harrisii*). The Julia Creek dunnart is listed in the IUCN Red List as Near Threatened (Burnett & Winter 2019) and has vanished from much of its previously known range (Woolley 2015). Captive breeding programs are therefore necessary if the species is to be successfully reintroduced into the wild (Woolley 2015). Crucially, understanding the genetic fitness of these animals would form an important part of conservation management plans for the species' long-term survival.

The Tasmanian devil already exhibits low genetic diversity due to the effects of several historic bottlenecking events caused by anthropogenic and environmental factors (Brüniche-Olsen et al. 2014). Unfortunately, the species is currently facing another bottlenecking event due to the impacts of Tasmanian devil facial tumour disease (Brüniche-Olsen et al. 2014). Part of the Tasmanian devil management strategy involves the maintenance of captive outbred populations until the disease is brought under control. Understanding and maintaining the genetic diversity of the species is critical to these efforts (Patchett et al. 2020).

3 METHODOLOGY

3.1 SAMPLE ACQUISITION

Ear tissue samples were collected from a representative sample (n = 100) of deceased specimens from the captive HVWC fat-tailed dunnart colony. These samples were chosen at

random from the deceased specimens, with no prior knowledge of the relatedness of the individuals chosen or which breeding generation they belonged to. Additionally, three tissue samples from wild-caught specimens were sourced from the Queensland Museum's collection. The exact type of tissue provided by the museum was not specified but was likely to be liver or muscle tissue (Dr Jessica Worthington Wilmer 2021, pers. comm., 1 March).

The tissue samples from the HVWC specimens were collected from the lower left earlobe using a standard 2 mm ear punch. Samples were stored in 70% ethanol in 1.5 mL biosphere microtubes at approximately 25°C. All equipment used was cleaned in household bleach between each biopsy to prevent cross-sample contamination (Fig. 2).

Figure 2: Biopsy sampling equipment used to take ear tissue biopsies from HVWC fat-tailed dunnarts.

Samples were labelled according to the identification system presently in use at HVWC. For example, ♀G1202 where '♀' identifies gender, 'G' identifies the allocated breeding group and

‘1202’ is a unique identifier. Refer to Appendices 1 and 2 for a full list of specimens included in the sample set.

3.2 DNA EXTRACTION

An initial trial was undertaken to compare two methods of DNA extraction for quantity and purity of yield. The methods trialled were: 1) a salting out protocol as described by Sunnucks and Hales (1996); and 2) Qiagen DNeasy® Blood & Tissue kits (69506) (DNeasy), prepared according to the manufacturer’s instructions.

Twelve biopsy samples were selected from the sample pool of HVWC specimens. Replicate biopsies were taken to give two identical sample sets. DNA extraction and analyses were carried out at The University of Queensland, Gatton campus.

Spectrophotometer analyses (absorbances at 260 and 280 nm) were conducted on all specimens in order to confirm the most effective method of DNA extraction, as well as to compare the quantity and purity of the DNA yield of all samples.

3.3 POLYMERASE CHAIN REACTION

3.3.1 Amplification of target loci

Reaction buffer trial

A polymerase chain reaction (PCR) was attempted targeting the following loci: ω-globin within the nuclear genome; two loci within the mitochondrial D-Loop control region (*CR*); and cytochrome *c* oxidase (COX). Two reaction buffers were trialled: 1) 2 x Phire Hot Start II PCR Master (ThermoFisher Scientific) (Phire) and 2) Amplitaq Gold 360 Master Mix DNA

Polymerase (ThermoFisher Scientific) (Amplitaq Gold). Primer sequences were derived from previous studies that successfully used them to amplify the target loci (Table 1).

Table 1: Primers, primer sequences and published annealing temperatures used to target the following loci: ω-globin; mitochondrial D-Loop control region; and cytochrome c oxidase.

Locus	Primer	Primer Sequences	Published annealing temp (°C)	Reference
ω-globin	G314 (F)	5'-GGA ATC ATG GCA AGA AGG TG-3'	48	Blacket et al. (2006b)
	G424 (R)	5'-CCG GAG GTG TTY AGT GGT ATT TTC-3'		Blacket et al. (2006b)
D-Loop	M20 (F)	5' -CCT CAC CAT CAG CAC CCA AGC- 3'	48	Cooper et al. (2000)
Control	M119 (R)	5' -TGC TGA TCT CTC GTG AGT TG-3'		Cooper et al. (2000)
Region				
D-Loop	M234 (F)	5' -ATA AGA CAT GCA GAT CAT TAC GCT A-3'	52	Cooper et al. (2000)
Control	M119 (R)	5' -TGC TGA TCT CTC GTG AGT TG-3'		Cooper et al. (2000)
Region				
Cytochrome	LCO1490	5'-GGT CAA CAA ATC ATA AAG ATATTG G-3'	40	Hebert et al. (2003)
c Oxidase	HCO2198	5'-TAA ACT TCAG GGT GAC CAA AAA ATC A-3'		Hebert et al. (2003)

Primers were ordered from Sigma-Aldrich and made up to 100 µM stock according to the supplier's instructions. Working stocks of 10 µM were then prepared. Initial optimisations were conducted in two 13 µL reactions. The first sequencing reaction consisted of: 6.25 µL 2 x Phire Mix reaction buffer; 0.25 µL each of 10 mM forward and 10 mM reverse primers; 5.25 µL distilled H_2O; and 1 µL DNA template. The second sequencing reaction was identical to the first except that 6.25 µL Amplitaq Gold reaction buffer was used instead of Phire. A nil-template control sample was included for each locus.

The PCR was carried out in a SimpliAmp™ thermal cycler (ThermoFisher Scientific) using a cycling protocol of 94°C for 3 min; 35 cycles of 95 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min; final extension at 72 °C for 7 min and 1 cycle of 25 °C for 1 s. The PCR products were electrophoresed on a 1.5% agarose gel cast and run in 0.5 x TBE and stained with ethidium bromide to test for presence and quality of the amplification. Migration rates through the gel were measured using GeneRuler 100 bp DNA ladder (ThermoFisher Scientific).

Second reaction buffer trial

Another PCR trial was run to compare the Phire and Amplitaq Gold reaction buffers a second time and confirm the results of the first amplification. In the second trial, only the COX gene was targeted since this produced the best amplification in the first trial. A sample size of n = 8 was selected.

Two 25 µL reactions were prepared for the COX locus. Sequencing reaction 1 consisted of: 12.5 µL 2 x Phire Mix; 0.5 µL 10 mM of the forward primer LCO1490; 0.5 µL 10 mM of the reverse primer HCO2198; 10.5 µL distilled H_2O; and 1 µL DNA template. Sequencing reaction 2 was identical to sequencing reaction 1, except that 12.5 µL Amplitaq Gold was used in place of Phire. A nil-template control sample was prepared for each reaction.

The same PCR cycling protocol was used as in the first reaction buffer trial. The PCR products were electrophoresed on a 1.5% agarose gel cast and run in 0.5 x TBE and stained with ethidium bromide to test for presence and quality of the amplification. Migration rates through the gel were measured using GeneRuler 100 bp DNA ladder.

3.3.2 Polymerase chain reaction optimisation

Some mild sub-banding of the COX gene was observed during the initial PCR trials, so a PCR optimisation was carried out to determine the best annealing temperature for this locus. A single sample of DNA template was tested across a temperature gradient ranging between 48-65°C. A master mix for eight 12.5 µL PCRs was prepared, each consisting of: 6.25 µL 2 x Phire Mix; 0.25 µL 10 mM of forward primer LCO1490; 0.25 µL 10 mM of reverse primer HCO2198; 5.25 µL H_2O; and 0.5 µL DNA template. No nil-template control template was prepared.

PCR optimisation was conducted in a gradient PCR machine using the following cycling protocol: 94°C for 3 min; 35 cycles of 95°C for 30 s, 48-65°C for 30 s, 72°C for 1 min; final extension at 72°C for 7 min; and 1 cycle of 25°C for 1 s. The following temperature gradients were used for the annealing stage: 48.0°C, 49.2°C, 51.2°C, 54.5°C, 58.3°C, 61.5°C, 63.7°C, and 65.0°C. Gradient stops were pre-determined by the thermocycler after setting the extremes at 48°C and 65°C.

A 5 µL sample of each of the PCR products was electrophoresed on a 1.5% agarose gel cast and run in 0.5 x TBE and stained with ethidium bromide to test for presence and quality of the amplification. Migration rates through the gel were measured using GeneRuler 100 bp DNA ladder.

3.3.3 PCR amplification of cytochrome *c* oxidase

PCR amplification of COX was conducted on DNA extracted from 32 individuals. The sample set was divided into two batches of sample size n = 14 and n = 18. In the second set of amplifications the last sample from the first set was reamplified to confirm consistency of the reaction across both batches.

A 25 µL PCR was conducted using: 12.5 µL of 2 x Phire mix; 0.5 µL 10 mM of the forward primer LCO1490, 0.5 µL 10 mM of the reverse primer HCO2198, 10.5 µL distilled H_2O and 1 µL DNA template. A nil-template control sample was prepared for each reaction.

PCR amplification was carried out using the following cycling protocol: 94°C for 3 min; 35 cycles of 95°C for 30 s, 52 °C for 30 s, 72 °C for 1 min; final extension at 72 °C for 7 min; and 1 cycle of 25°C for 1 s.

The PCR products were electrophoresed as described above.

3.4 DNA SEQUENCING

The 14 samples comprising batch 1 were selected for DNA sequencing. The PCR products were purified using SureClean Plus as per the manufacturer's instructions. Briefly, these were mixing 20 µL of PCR product from the previous step with 4 µL pink co-precipitant in a 1.5 mL microtubes, adding 24 µL of SureClean Plus, vortexing for 30 s, then incubating at 25°C for 20 min. The tubes were then centrifuged for 10 min at 14,000 rpm. The supernatant was pipetted off and 48 µL of 70% ethanol added. The product was then vortexed for 10 s and centrifuged for 10 min at 14,000 rpm. The ethanol was pipetted off and the product left to air dry for approximately 5 min until all the ethanol had evaporated. The product was resuspended in 34 µL H_2O. This product was then split (17) µL into two tubes and 4.5 µL of 8 µM forward and reverse primer was added to each tube respectively to obtain the forward and reverse sequences. Tubes were consigned to Macrogen Inc. for Sanger sequencing.

The sequenced data was received from Macrogen Inc. in the form of chromatogram files. These were analysed using FinchTV v.1.4.0, MEGA-X v.10.2.1 (Kumar et al. 2018) and NCBI (National Center for Biotechnology Information 2021) BLAST (Basic Local Alignment Search

Tool). MEGA-X is freely available software that enables comparative analyses of molecular sequences (Kumar et al. 2018). FinchTV is freely available software developed by Geospiza Inc. and enables viewing of sequenced data as a chromatogram (Digital World Biology 2019). NCBI BLAST is a sequence alignment tool that allows the user to search for regions of similarity between nucleotide or protein sequences.

3.5 PRIMER DESIGN AND MICROSATELLITE IDENTIFICATION

In silico analyses

Early access to a draft, non-annotated fat-tailed dunnart genome was obtained with permission, via personal communication, from the lab of Professor Andrew Pask, The University of Melbourne. This file contained 719 scaffolds, representing individual sections of contiguous sequences (contigs). These contigs ranged in size from a maximum length of 442.9 million bp to a minimum of 518 bp. The average contig length was 3.95 million bp. These contigs could represent DNA on different chromosomes, or DNA segments from any given chromosome separated by repeat regions, which cannot be stitched together without long-read scaffolds. Stitching together of the scaffolds was not attempted as it is out of scope for this project. The draft genome was uploaded to Krait v.1.3.3 as a FASTA file. Krait is freely available software designed for genome wide investigation of microsatellites. The program can be used to search for perfect, imperfect and compound microsatellites, and to develop primers for those microsatellites (Du et al. 2018)

Once uploaded to Krait, the data were interrogated for perfect microsatellites consisting of sequences of seven or more repeats of tri-nucleotide motifs. A primer design search was run using the program's default settings, excepting the following changes: the primer product size

range was set to 100 – 150 bp; the minimum primer melting temperature was set at 62°C; the optimum primer melting temperature at 64°C; and the maximum primer melting temperature at 66°C.

The output file was exported into Microsoft Excel as a *csv* file. A range of prospective loci were selected based on a product size of 100 bp, and were chosen from different contigs, or different regions of the contigs, to control against linkage disequilibrium. The 100 bp product size was selected because a change in variance of only a few base pairs is more likely to be detected in a smaller than a larger product. Smaller products are also amenable to quantitative PCR (qPCR) for microsatellite discrimination. The selected primer sequences were screened by eye and discarded if they contained any obvious microsatellites within the primer sequence. Each pair of primers was then screened for self-annealing and hairpin formation using Sequence Manipulation Suite v.2 (Stothard 2000). Using this process, a sample set of 20 was selected for PCR amplification.

PCR amplification

The selected primers were ordered from Sigma-Aldrich. The lyophilised primers were rehydrated to 100 µM stock solution as per the manufacturer's instructions. A 1:10 dilution in H_2O was then prepared as a 10 µM working stock. Amplification of the selected loci was attempted using standard PCR. Each locus was subjected to three trials, using different DNA template for each trial. Three 13 µL reactions were prepared for each set of primers. Each reaction consisted of 6.25 µL 2 x Phire mix; 0.625 µL each of 10 µM forward and reverse primers; 5.25 µL H_2O; and 1 µL DNA template. A nil-template control was prepared for each set of reactions. The PCR cycling protocol was: 95°C for 5 min; 35 cycles of 95 °C for 20 s, 57 °C for 30 s, 72 °C for 20s; final extension at 72 °C for 7 min; and one cycle of 25 °C for 1 s.

The PCR products were electrophoresed on a 0.5 x TBE, 2% agarose gel stained with ethidium bromide to test for presence and quality of the amplification. Migration rates through the gel were measured using GeneRuler 100 bp DNA ladder.

Any microsatellites identified during this PCR were screened for possible allelic diversity and heterozygosity. A total of 18 DNA template samples were used, comprising 15 of the captive specimens from the HVWC colony and the three wild specimens sourced from the Queensland Museum. Amplification of the identified microsatellites was done using a standard PCR. A 13 µL reaction was prepared for each sample consisting of: 6.25 µL 2 x Phire Mix: 0.625 µL each of 10 µM forward and reverse primer; 5.25 µL H_2O; and 1 µL DNA template. A nil-template control sample was also prepared. The PCR cycling protocol was 95°C for 5 min; 35 cycles of 95°C for 20 s, 57°C for 30 s, 72°C for 20s; final extension at 72°C for 7 min; and one cycle of 25°C for 1 s.

The PCR products were electrophoresed on a 0.5 x TBE, 2% agarose gel stained with ethidium bromide to test for presence and quality of the amplification. Migration rates through the gel were measured using GeneRuler 100 bp DNA ladder.

4 RESULTS

4.1 SAMPLE ACQUISITION

Ear tissue biopsies from 100 deceased fat-tailed dunnarts were collected from the HVWC on the 29th and 30th October 2020 and stored in 70% ethanol at approximately 25°C. Tissue samples from three wild-caught dunnarts were sourced from the Queensland Museum. Two of the wild-caught specimens originally came from the Wallumbilla district, Queensland and

the third from Diamantina Lakes National Park, Queensland. These samples were also stored in ethanol.

4.2 DNA EXTRACTION

The salting out protocol yielded a higher quantity of DNA. However, the overall purity of DNA using this method was lower when compared to the Qiagen DNeasy® Blood & Tissue kits. Sample set 1 (salt extraction) had an average quantitative yield of 29.36 ng/µL compared to 4.49 ng/µL from the DNeasy kits. Anything over 10 ng/µL is considered good yield.

DNA purity is measured as an absorbance ratio A_{260}/A_{280}. A range between 1.6 – 2.0 is considered to indicate a good level of purity. Anything outside this range indicates contamination, for example, by proteins. Sample set 1 had an average absorbance ratio of 3.10 and sample set 2 an average absorbance ratio of 2.18. The quantity and purity of DNA obtained from each sample set was confirmed by spectrophotometer analyses (Table 2).

Table 2: Results for 12 DNA template samples comparing two methods of DNA extraction, sample set 1) a salt extraction protocol and sample set 2) Qiagen DNeasy® Blood & Tissue kits. Comparison was on the basis of DNA yield (ng/µL) and purity (A_{260}/A_{280}).

Salt extraction protocol			Qiagen DNeasy® Blood & Tissue Kits		
Sample no.	ng/µL	A_{260}/A_{280}	Sample no.	ng/µL	A_{260}/A_{280}
1	19.3	3.95	1	3.4	1.75
2	27.6	2.51	2	7.6	1.67
3	71.8	2.86	3	6.5	1.53
4	31.4	2.80	4	5.2	1.99
5	17.5	2.84	5	4.9	2.06
6	28.1	2.65	6	4.5	2.13
7	23.1	3.85	7	3.7	1.75
8	24.3	2.65	8	1.2	3.15
9	20.9	2.47	9	3.8	2.19
10	22.7	3.16	10	5.3	2.01
11	50.4	2.74	11	4.9	3.32
12	15.2	4.73	12	2.9	2.71

Although the salt extraction protocol yielded a consistently higher quantity of DNA, the A_{260}/A_{280} ratio showed consistently high levels of co-extraction of non-nucleic acid components. Based on these results, the DNeasy kits, which yielded consistently higher DNA quality, were used for all remaining DNA extraction. Although the quantity of DNA per sample was low, it was still sufficiently high to run a successful PCR.

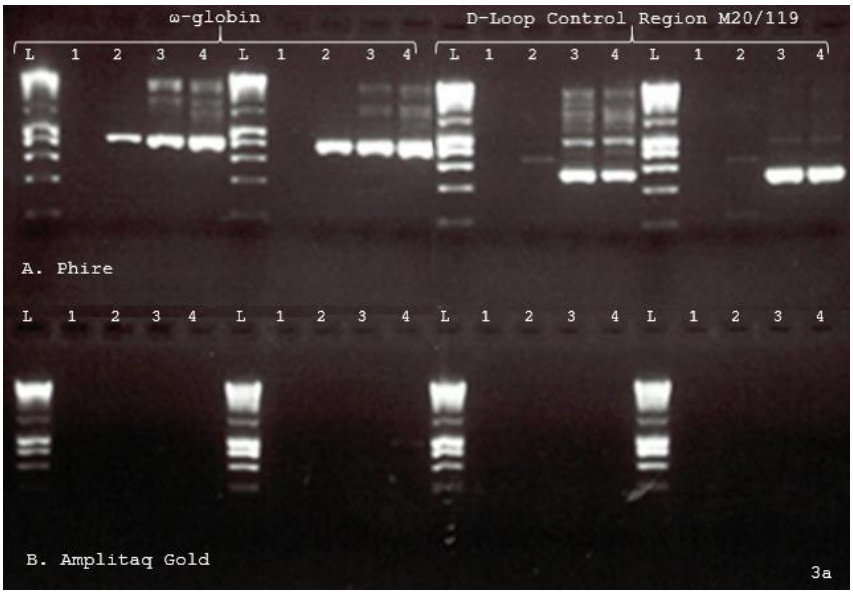
DNA extraction was therefore continued on the full sample set using the DNeasy kits. DNA yield and purity were confirmed for all specimens via spectrophotometer analyses. The results of the spectrophotometer analyses for all samples from the HVWC colony are listed in Appendix 1, and those for the wild specimens from the Queensland Museum in Appendix 2.

4.3 POLYMERASE CHAIN REACTION

4.3.1 Amplification of target loci

Reaction buffer trial

Results of the initial amplification of the target loci from the fat-tailed dunnart DNA are shown in Figure 3a) and b). The top rows of Fig 3a) and b) show the results using the Phire reaction buffer and the bottom rows the results using Amplitaq Gold. Phire delivered good results for all loci, successfully isolating the following sequences: 750 bp ω-globin; 300 bp D-Loop Control Region M20/119; 200 bp D-Loop Control Region M254/119; and 800 bp Cytochrome *c* oxidase. Amplitaq Gold failed to amplify any of the target loci.



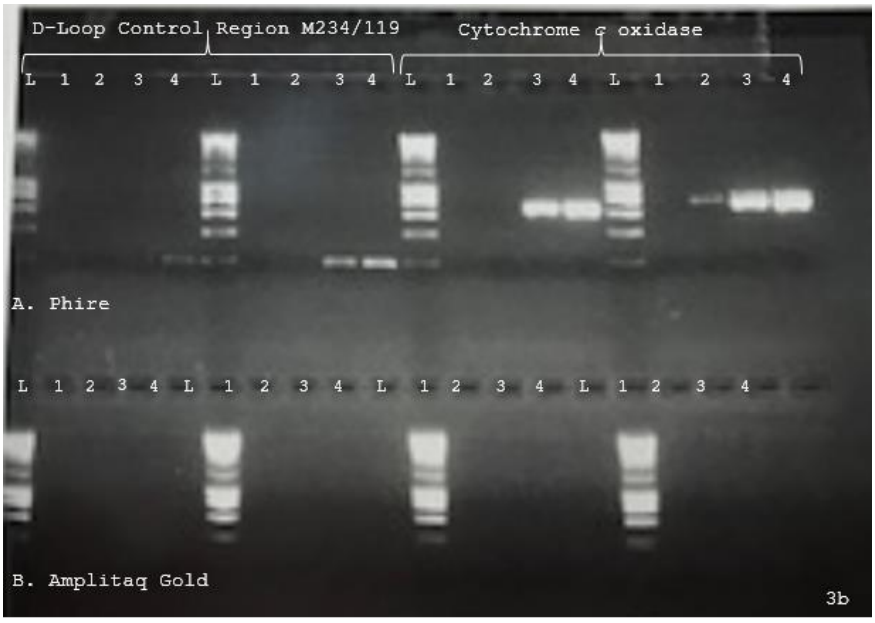


Figure 3: Amplification of target loci ω-globin, D-Loop Control Region M20/119, D-Loop Control Region M234/119 and Cytochrome c oxidase trialling Phire and Amplitaq Gold reaction buffers. Row A in each image shows the results for Phire and row B the results for Amplitaq Gold. 'L' signifies the DNA ladder and well number 1 in each series is the nil-template control.

Second reaction buffer trial

The results of the second reaction buffer trial are shown in Figure 4. Strong amplification of approximately 800 bp of COX was achieved using Phire, while no amplification was achieved using Amplitaq Gold. Phire was therefore used in all remaining experiments.

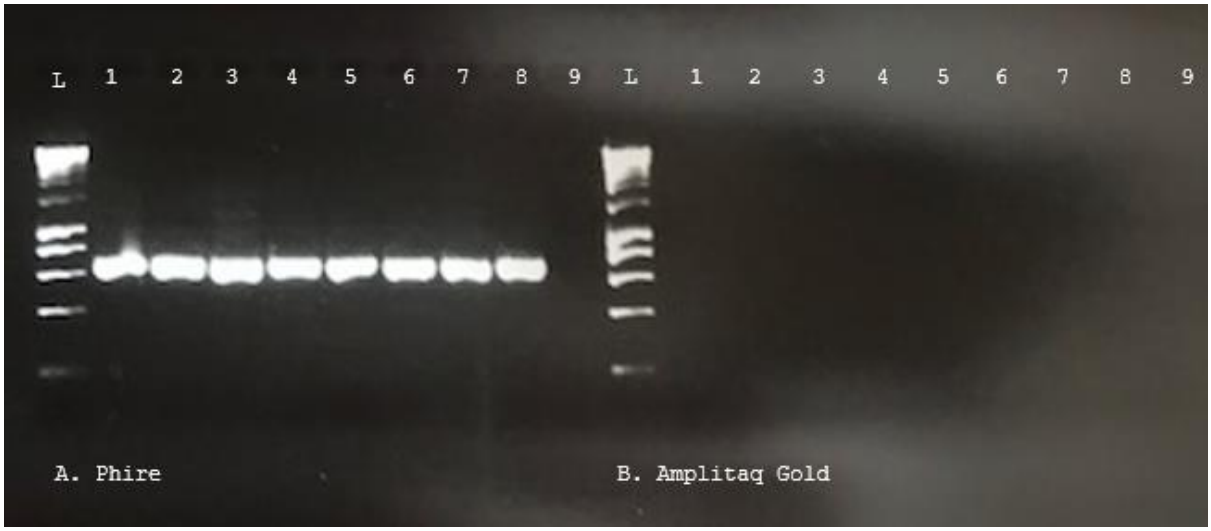


Figure 4: Second trial amplification of Phire and Amplitaq Gold confirming the results of the first trial. In this trial, only the COX gene was amplified. 'L' signifies the DNA ladder and well 9 in each series is the nil-template control.

4.3.2 PCR optimisation

The results of the PCR optimisation of the COX gene are shown in Figure 5. Strong amplification with minimal non-target banding was achieved at 51.2°C. An annealing temperature of 52°C was therefore used for the remaining experiments with the COX locus.

Figure 5: Results of PCR optimisation using a graduated annealing temperature. 'L' signifies the DNA ladder. A nil-template control was not included in this experiment.

4.3.3 PCR amplification of cytochrome *c* oxidase

The results of the amplification of the COX gene are shown in Figure 6a and 6b. Figure 6a shows the results for batch 1 and Figure 6b for batch 2. Good amplification at 800 bp was achieved for all samples, except for well 4 in batch 2 which failed to amplify.

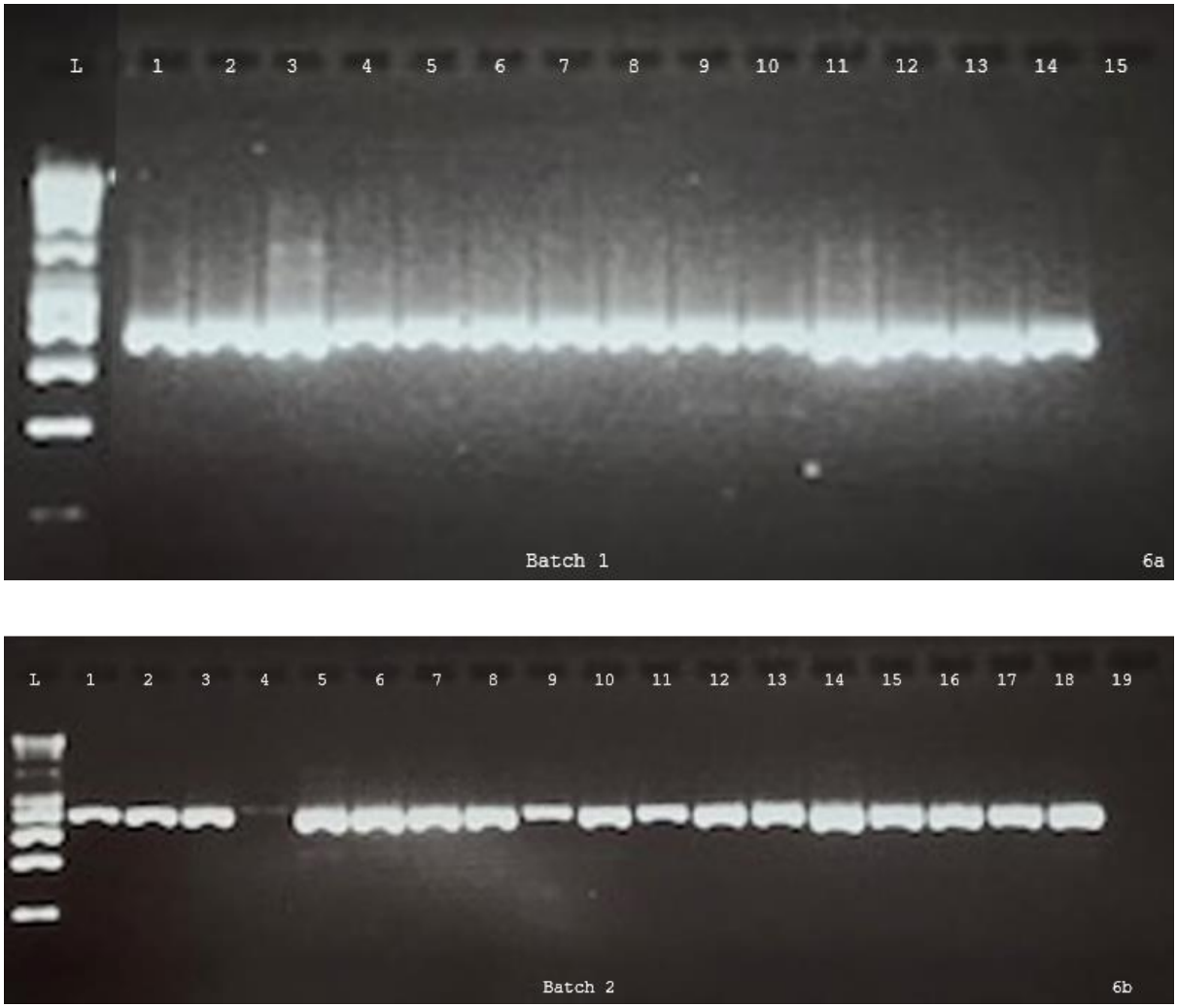


Figure 6: Cytochrome c oxidase amplification showing strong amplification in all wells except well 4 in batch 2. 'L' signifies the DNA ladder. Well 14 in batch 1 and well 1 in batch 2 are replicate samples. Well 15 in batch 1 and well 19 in batch 2 are the nil- template controls.

4.4 DNA SEQUENCING

There were 13 forward and 11 reverse sequences found that were of high quality. The combined total of 24 sequences were used to generate a consensus sequence in MEGA-X. All potential variants were cross-checked against the chromatogram and manually edited to remove any sequencing errors.

The consensus sequence was analysed in BLAST. The BLAST results indicated 641/646 (99%) matches to GenBank accession number AY795974.1, *S. crassicaudata* mitochondrion genome (Phillips et al. 2006). The 5 differences were checked against the chromatogram and found to be genuine variants, not sequencing error.

The consensus sequence was also compared with the draft genome provided by the Pask lab. The BLAST results indicated 100% match between our consensus sequence and the draft genome.

No variation was found at this locus for the samples analysed from the captive fat-tailed dunnart population.

4.5 PRIMER DESIGN AND MICROSATELLITE IDENTIFICATION

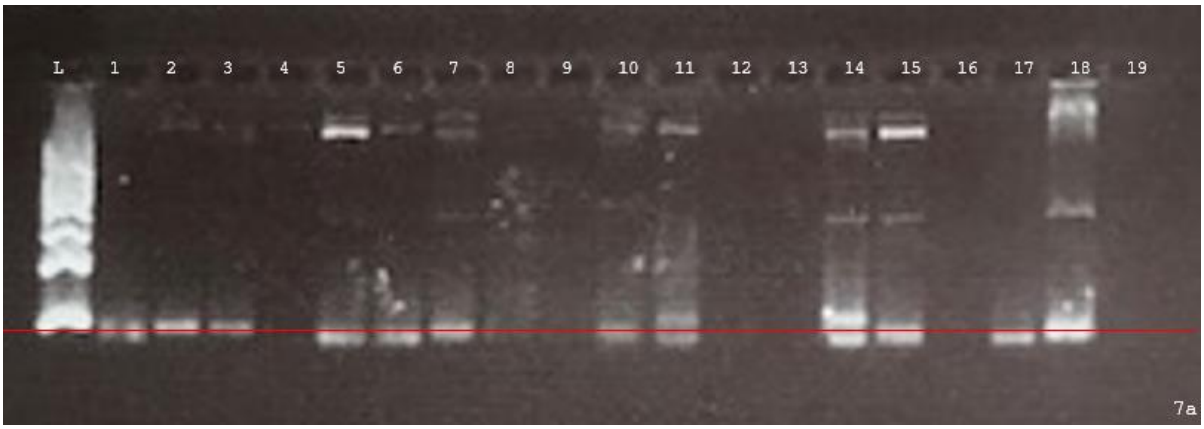
In silico analyses

The selected primer sequences are listed in Table 3. Of the 20 loci that were trialled for microsatellite identification, one successfully amplified. This was the microsatellite located within contig 19.

Table 3: Forward and reverse primer sequences selected for identification of microsatellites within the fat-tailed dunnart genome. Also shown are the contig (sequence) within which each microsatellite is located, its tri-nucleotide motif and the number of motif repeats

Contig	Motif	Repeat	Forward primer	Oligo name (fwd)	Reverse primer	Oligo name (rev)
2	GAG	10	AGGAGGGGGAGGAAGAGGGG	2a F	TCCTTCTCCCCCTCCTCCCC	2a R
2	TTG	8	ACTGAACAAACTGCCTAAAAACACAGC	2b F	ATGTCATGAGCCTCTAATGGAAGCC	2b R
4	TGT	14	TCTACAGGATTGTTCCAAGGCAGG	4a F	ACTGCTCAAGGAGTCCAAGCC	4a R
4	CCT	10	GTGACGCAGAGGTGTTCGCC	4b F	GGGAGGGGGAAAGGAGGTGG	4b R
8	TTG	9	TTTCTGAGGCAGCCCATCCC	8a F	TGGCAAAACTGGGAACAATAGATGGG	8a R
11	TTA	15	TTGTTTCAGGTAGGAGATGGGAGC	11a F	TCACTTAACCCCTATTGCCTCAGC	11a R
11	ATG	8	CTCCCTAGTTCTTATTTGACGTCTCCC	11b F	TAGGCTGCTCTGCTACCACACC	11b R
12	GTT	11	CGTTTGAAAGCAAGTTTCTCCAGGG	12a F	TGTGACTTGCCCGGTGTTGG	12a R
15	CAT	8	GGCTCCCTCACATTGTAAAGAAAGACC	15a F	GCCTTGTCAAATTTTCATGGTGGTGC	15a R
17	AGG	8	GGGAGGACATGCTGCAAGGC	17a F	TACCCCTCTTCCCCAGGAGC	17a R
19	AAT	8	AGACAGAGGTTAAGTGACTTTCCAGG	19a F	GGAAGACTTGTGGTCCTGCTTTGG	19a R
20	AAC	10	GTCCAGGAGTTATAGAGCATGAGGGG	20a F	GGCTTTGAGATGATCAGTGTGGTGG	20a R
21	TAG	11	GTAGTAGTGGGGTGGGTGGTGG	21a F	AACAGCAACAACAAAAATTACAGCAGC	21a R
23	GGA	9	AGAGAAGGGGGAGGGGGAGG	23a F	ACCCTGTCTGCTGCAACTGC	23a R
25	TTA	10	GCCTGTCTTCCCACAGTACCTCC	25a F	TGCTTGTGACCAGACTATCAGATTAGC	25a R
48	CTC	9	TAGTGCCTGGAGCTAGGGGC	48a F	AGATGCCCTGGCCTCATGGG	48a R
49	TTA	9	CAGAGGGAGGTTTCAACATTCAGTGG	49a F	ACCTCAGCTACTTTGATCCAACAGG	49a R
53	AAC	11	ACCAACACCAAACACCACCACC	53a F	TGGTGTTGTTGTTTGTTGTTTGGTGG	53a R
58	TTG	10	ATGGTGGCTTTGAGCTGACTTGG	58a F	ACAAAACCTGCTGGGGATGGC	58a R
59	AAT	18	TGACCTGTTTGGGGCTTCAGC	59a F	GAGTCAAGCCCACATTTCCCCC	59a R

Further amplification of this loci using 18 individual DNA templates revealed a degree of allelic diversity and heterozygosity at this locus. Of the 18 samples selected, five amplified at 97 bp, five at 100 bp and two were heterozygous for 97 and 100 bp (Fig. 7a and 7b).



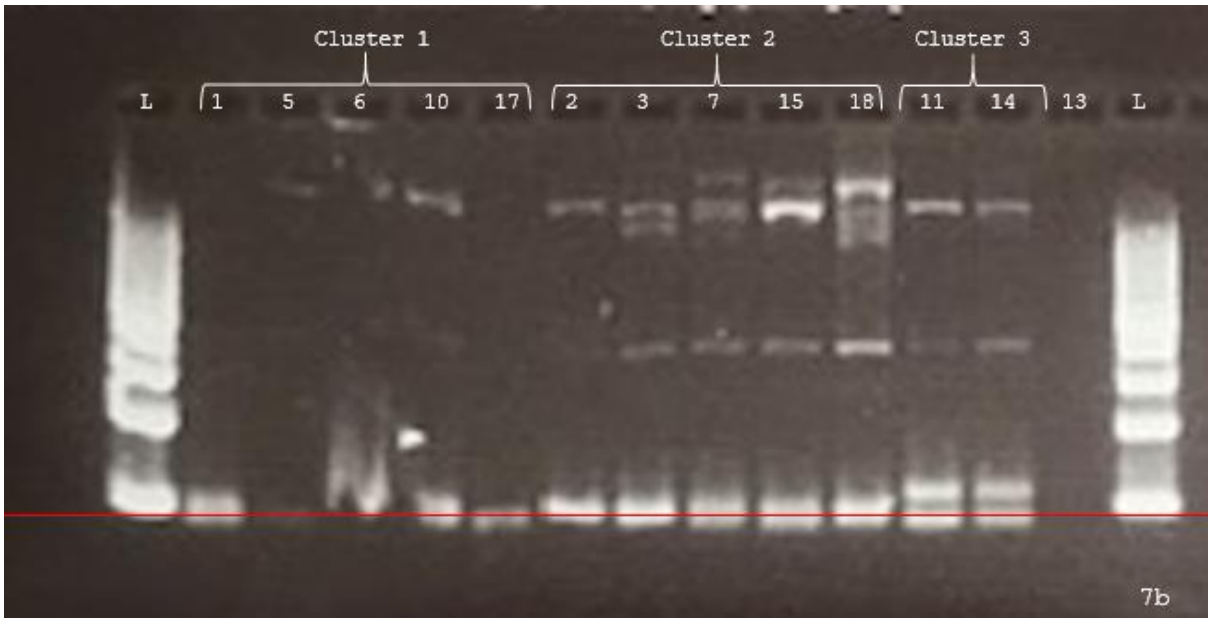


Figure 7: Results of the amplification of the successfully isolated microsatellite. Figure 7a shows the initial amplification using 18 individual DNA template samples. Samples 1-15 are from the captive colony and samples 16-18 are the wild specimens. The red line shows approximately 100 bp amplification. The samples in wells 1, 5, 6, 10 and 17 have amplified to 97 bp. The samples in wells 2, 3, 7, 15 and 18 have amplified to 100 bp. The samples in

wells 11 and 14 show heterozygosity for 97 and 100 bp. The samples in wells 4, 8, 9, 12, 13 and 16 failed to amplify. 'L' signifies the DNA ladder and well 19 is the nil-template control.

Figure 7b shows the samples that successfully amplified re-grouped in stepwise fashion. The first cluster shows the samples that amplified at 97 bp, the second cluster shows the samples that amplified at 100 bp, and the third cluster shows the samples that are heterozygous for 97 and 100 bp. 'L' signifies the DNA ladder, and well 13 is the nil-template control.

5 DISCUSSION

5.1 PROJECT AIMS AND OBJECTIVES

The principal aim of this project was to investigate and develop methods of determining genetic variation within a species, using a captive colony of fat-tailed dunnarts as a model. This involved two main objectives: 1) testing existing well-established methods of determining genetic variation through PCR amplification and sequence analyses of candidate mitochondrial and nuclear genome loci; and 2) targeting and amplifying nuclear microsatellite markers within the fat-tailed dunnart genome through a combination of *in silico* and *in vitro* analyses.

5.2 FINDINGS

Objective 1: PCR amplification and sequence analyses of candidate mitochondrial genome loci

Meeting this objective involved successful extraction of DNA from ear tissue biopsies of a representative sample of fat-tailed dunnart specimens from the HVWC captive colony and

from unspecified (likely muscle or liver) tissue from the wild fat-tailed dunnart specimens supplied by the Queensland Museum. Trials of two methods of DNA extraction and spectrophotometer analyses confirmed that DNA of sufficient quantity and purity could be extracted from most of the specimens.

Once DNA extractions had been completed for all biopsy samples, a series of PCR amplifications were carried out to attempt to successfully isolate the following candidate mitochondrial and nuclear loci: cytochrome *c* oxidase (COX); D-Loop control region (*CR*); and ω-globin. These loci were selected based on evidence in the literature demonstrating their efficacy in determining genetic variation within closely related populations (Cooper et al. 2000; Hebert et al. 2003; Blacket et al. 2006b). Subsequent trials with two different reaction buffer enzymes were successful in targeting all four loci. The most successful amplification was of the COX gene; therefore these samples were selected for sequencing.

In silico analyses of the sequenced samples showed they aligned perfectly, confirming that there was no variation at this locus. Comparison of the sequenced samples with the draft genome provided by the Pask lab likewise confirmed no variation, however comparison with a published fat-tailed dunnart genome in BLAST (Phillips et al. 2006) found slight variation (99% match) between our consensus sequence and the published genome. As Phillips et al. (2006) do not identify the source of their fat-tailed dunnart specimen, we cannot make any assumptions about any degree of relatedness between this specimen and those in the HVWC captive colony, however it is fair to assume that there is a degree of variation at the COX locus within the fat-tailed dunnart population as a whole. This may lend support to findings by Hope et al. (1986) and Cooper et al. (2000) of two genetic subclades within this species.

The results from the sequencing of the COX gene would appear to indicate little to no variation at this particular locus. Although the representative sample was small, it was randomly selected with no prior knowledge of the level of relatedness between the sample specimens.

Objective 2: Target and amplify microsatellite markers

In silico analyses of the draft fat-tailed dunnart genome successfully identified perfect tri-nucleotide microsatellites and designed primers for those microsatellites. Out of the pool of possible primers, 20 were selected as suitable candidates for further PCR amplification. The selected primers were screened by eye for any obvious microsatellites and screened *in silico* for self-annealing or hairpin formations. Primers that failed any of these criteria were not included in the final sample set.

PCR amplification was attempted in triplicate on all 20 loci, with the result that one, located within contig 19 of the draft genome, amplified successfully. The successful amplification, identified for ease of reference as microsatellite 19, was subjected to a second PCR amplification. In this amplification, 18 DNA template samples were used, 15 from the captive specimens and three wild specimens. In this second amplification clear evidence was found of both polymorphism and heterozygosity at the target locus of the order of three base pairs difference. Furthermore, this degree of polymorphism was successfully detected through electrophoresis on an agarose TBE gel, rather than on a more toxic acrylamide gel.

Polymorphism and heterozygosity were detected within the captive population, clearly indicating a degree of variation at this locus. This finding is significant as it shows that the methods used in this project to identify and amplify a microsatellite marker are demonstrably successful. Moreover, comparison of the results of objectives 1) and 2) demonstrates no

variation in the mitochondrial genome, but evidence of variation within the nuclear genome. This is likely a factor of the faster evolution and higher substitution rates that are a known characteristic of microsatellites (Schlötterer 2004; Abdul-Muneer 2014; Vieira et al. 2016). It may also be indicative of the tendency of loci within the mitochondrial DNA to demonstrate reciprocal monophyly sooner than nuclear alleles (Moritz 1994). The fact that the sequencing of the captive specimens demonstrated no variation indicates a higher degree of relatedness between these specimens, particularly when compared with the published genome (Phillips et al. 2006) and the draft genome provided by the Pask lab.

Genotype variation was successfully observed at a single microsatellite locus. This variation was observed between members of the captive colony as well as between the captive specimens and the wild specimens. The identification of this locus warrants further validation, but, importantly, shows that successful development of microsatellite loci was achieved in this project, which has provided a pathway for further investigation of population genetics in the fat-tailed dunnart and other marsupial species. To our knowledge, this is the first time a microsatellite has been successfully isolated within the fat-tailed dunnart genome.

5.3 LIMITATIONS

Objective 1: PCR amplification and sequence analyses of candidate mitochondrial genome loci

At the time we sequenced the COX locus, we did not have access to the wild specimens, so could not use these as a point of comparison. It would be a useful exercise to sequence the wild specimens as well particularly given that we know the geographical origin of these specimens (Wallumbilla district, Queensland and Diamantina Lakes National Park

Queensland). This would give an indication of what level of variation exists at the COX locus between specimens known to be sourced from geographically disparate areas. It would also be a useful point of comparison with the published genome.

As the sample size used to sequence the captive HVWC specimens was also fairly small, it would be beneficial to replicate these experiments with a larger sample size which included tissue samples from living specimens. This would allow us to confirm whether the lack of variation at the COX locus is indeed universal throughout the captive colony.

Objective 2: Target and amplify microsatellite markers

The initial screening in Krait to identify microsatellites and primer sequences returned an extremely large number of hits. Even limiting the search to just trinucleotides with a product length of 100-150 repeats returned 39,752 results. Isolating 20 candidate sequences from this list, even with manual screening for obvious microsatellite sequences and further *in silico* analyses to eliminate sequences with self-annealing or hairpin formations, was therefore something of a speculative undertaking. However, the results have demonstrated the efficacy of this method.

There were also some problems with the first attempt at electrophoresis of the amplified microsatellite product. The gel did not run very well, possibly due to a loss of ionisation in the TBE buffer. Despite this, the amplification of microsatellite 19 was clear enough to allow us to proceed with further trials of this locus, which ultimately proved successful.

6 CONCLUSION AND RECOMMENDATIONS

The findings in this project are significant for several reasons. The methods developed are demonstrably successful and can be used to develop a suite of microsatellite markers that will have application in further research, not only on the fat-tailed dunnart but also on other marsupial species. Further experimentation and replication of the methods used here is warranted to refine the techniques. Replication, with a larger DNA sample across a greater number of microsatellite loci, will give a clearer picture of the degree of heterozygosity and polymorphism present within the Hidden Vale captive colony. PCR optimisation is recommended in order to determine the optimum annealing temperature and so improve the clarity and accuracy of the results. Quantitative PCR and SYBR melt-curve analysis is recommended as a next stage for the project as a method to screen for allelic discrimination.

The importance of understanding and preserving the genetic diversity of the world's wildlife is steadily gaining traction. As molecular technology improves, so too does its accessibility and application to conservation strategies. More attention must be given to understanding the implications of the loss of genetic diversity, even in species considered common or Least Concern. This is particularly so given the increased pressure on the world's wildlife in the face of range extraction and population extinction. The events we are witnessing now may well prove to be the 'canary in the mine', a prelude to a potentially catastrophic cascade of species and biodiversity loss. The findings of this project are a contribution to that effort.

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8 APPENDICES

8.1 APPENDIX 1: SAMPLE SPECIMENS FROM THE HVWC CAPTIVE COLONY

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
1	2	F	G1411	08-Apr-19	1106	1142	G1413 (F) (Fu) G1415 (M)(Fu) G1410 (F)(Fu) G1527 (M)(HS)	0	19.3	3.95
2	1	M	G1104	23-May-18	984	880	G1113 (F) (HS) G1105 (M)(Fu)	0	27.6	2.51
3	2	F	R1497	28-Jun-19	Oy222	Oy232	R1520 (M)(HS) R1519 (M)(HS)	0	71.8	2.86
4	2	M	R1489	29-May-19	1077	1083	R1263 (M) (HS) R1492 (M) (Fu)	0	31.4	2.80
5	n/a	F	Y1377	n/a	n/a	n/a	0	0	17.5	2.84
6	2	M	O1399	29-Mar-19	Ob196	Oy179	O1393 (F)(Fu) G1512(F)(HS)	0	28.1	2.65
7	2	M	G1415	08-Apr-19	1106	1142	G1413 (F) (Fu) G1410 (F)(Fu) G1411 (F)(Fu) G1527 (M)(HS)	0	23.1	3.85
8	2	M	B1235	04-Feb-19	1168	1109	0	0	24.3	2.65
9	2	F	G1418	08-Apr-19	1182	1143	G1423 (M) (Fu)	0	20.9	2.47
10	1	M	Y1161	25-Jul-18	945	890	Y1162 (M)(Fu) Y1164 (M)(Fu)	Sire to Y1448 (M) Y1450 (M)	22.7	3.16
11	1	M	Y1162	25-Jul-18	945	890	Y1164 (M) (Fu) Y1161 (M)(Fu)	0	50.4	2.74
12	2	M	B1273	13-Feb-19	1149	1128	B1249 (M) (HS) B1271 (F) (Fu)	0	15.2	4.73

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
13	1	F	Y1160	23-Jul-18	1053	993	R1159 (M) (Fu) R1154 (F) (Fu)	Dam to R1485 (M)	6.5	1.90
14	1	M	R1150	18-Jul-18	955	950	R1142 (M)(HS)	Sire to G1405 (M) G1407 (M)	1.5	1.50
15		M	G1100	n/a	n/a	n/a	0	0	9.0	1.92
16	3	M	R1519	17-Jul-19	1295	Oy232	R1520 (M)(Fu) R1497 (F) (HS)	0	2.1	1.66
17	2	F	G1463	08-May-19	1183	1169	G1527 (M)(HD)	0	1.7	1.11
18	2	M	G1227	04-Feb-19	1125	1092	G1246 (M) (HS) G1241 (F) (HS) G1460 (M)(HS)	0	1.3	0.69
19	1	M	OB219	27-Aug-18	g117	r89	OB214(F)(Fu)	0	2.8	1.34
20	1	F	OB214	27-Aug-18	g117	r89	OB219 (M) (Fu)	Dam to Y1473 (F) Y1474 (F) Y1476 (M)	2.7	0.97
21	2	M	B1249	06-Feb-19	1148	1128	B1271 (F) (HS) B1273 (M) (HS)	Sire to B1533 (M)	2.1	1.37
22	2	F	B1313	27-Feb-19	1195	1129	B1311 (F)(Fu)	0	2.3	1.00
23	2	M	G1460	03-May-19	1220	1092	G1227 (M)(HS) G1246 (M)(HS) G1241 (F)(HS)	0	2.9	1.34
24	2	M	G1432	22-Apr-19	1203	1144	0	0	1.2	1.79
25	2	M	R1263	13-Feb-19	1076	1083	R1492 (M) (HS) R1489 (M)(HS)	0	1.9	1.09

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
26	1	M	B1185	06-Aug-18	1034	1009	B1183 (F) (Fu) B1223 (M) (HD) B1222 (M) (HD) B1187 (M) (Fu)	0	2.0	1.02
27	1	M	G1193	08-Aug-18	1062	1008	0	0	2.8	1.60
28	1	M	G1201	24-Aug-18	1069	1039	G1202 (M) (Fu)	0	7.3	1.64
29	3	F	Y1576	27-Jan-20	1240	1279	0	0	1.7	0.84
30	2	F	G1379	20-Mar-19	1132	1078	Y1279(M)(HS) Y1383 (F)(Fu) Y1380 (M)(Fu) Y1381 (M) (Fu) Y1379 (M)(Fu)	0	3.7	1.80
31	2	M	Y1381	20-Mar-19	1132	1078	Y1279(M)(HS) Y1383 (F)(Fu) G1379 (F)(Fu) Y1380 (M)(Fu) Y1379 (M)(Fu)	0	2.2	1.38
32	1	F	R1154	23-Jul-18	1053	993	Y1160 (F) (Fu) R1159 (M) (Fu)	0	2.6	1.58
33	2	M	G1246	06-Feb-19	1126	1092	G1227 (M) (HS) G1241 (F) (Fu) G1460 (M)(HS)	0	1.1	2.56
34	2	F	G1443	29-Apr-19	1216	1091	G1444 (F)(Fu) G1438 (M)(HS) G1434 (F)(HS) G1436 (F)(HS)	0	1.4	1.74
35	1	F	OR200	30-Jul-18	g107	781	OY222 (F)(HS)	Dam to B1503 (M)	2.5	1.79
36	1	M	G1122	04-Jun-18	1024	932	G1118 (F)(Fu)	0	0.5	2.27

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
37	2	M	Y1448	03-May-19	1138	1161	Y1450 (M) (Fu)	0	1.7	3.97
38		F	R1030	n/a	n/a	n/a	0	0	14.1	1.99
39	2	M	G1405	01-Apr-19	1205	1150	G1407 (M) (Fu)	0	4.1	2.25
40	2	F	B1311	27-Feb-19	1195	1129	B1313 (F)(Fu)	0	1.6	1.74
41	X	M	B1072	29-Mar-18	946	924	B1074 (M)(Fu)	0	2.9	1.96
42	1	M	R1159	23-Jul-18	1053	993	Y1160 (F) (Fu) R1154 (F) (Fu)	0	3.8	2.46
43	2	M	Y1380	20-Mar-19	1132	1078	Y1279(M)(HS) Y1383 (F)(Fu) G1379 (F)(Fu) Y1381 (M) (Fu) Y1379 (M)(Fu)	0	3.4	2.14
44	2	F	G1444	29-Apr-19	1216	1091	G1443 (F)(Fu) G1438 (M)(HS) G1434 (F)(HS) G1436 (F)(HS)	0	3.4	1.81
45	1	M	R1142	09-Jul-18	908	950	R1150 (M) (HS)	Sire to G1410 (F) G1411 (F) G1413 (F) G1527 (M) G1415 (M)	4.2	1.72
46	3	M	B1544	25-Nov-19	1486	1337	B1539 (F)(Fu)	N	2.3	1.72
47	1	M	B1222	17-Dec-18	1034	1027	B1183 (F) (HD) B1223 (M) (Fu) B1185 (M) (HD) B1187 (M) (HD)	0	1.3	6.78

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
48	2	F	G1413	08-Apr-19	1106	1142	G1415 (M)(Fu) G1410 (F)(Fu) G1411 (F)(Fu) G1527 (M)(HS)	0	2.3	2.41
49	3	M	R1520	17-Jul-19	1295	Oy232	R1519 (M) (Fu) R1497 (F)(HS)	0	2.6	1.41
50		M	G1136	n/a	n/a	n/a	0	0	2.6	1.63
51	2	F	Y1383	20-Mar-19	1132	1078	Y1279(M)(HS) G1379 (F)(Fu) Y1380 (M)(Fu) Y1381 (M) (Fu) Y1379 (M)(Fu)	0	3.8	1.95
52	1	M	G1105	23-May-18	984	880	G1104 (M)(Fu) G1113 (F)(HS)	0	1.6	5.89
53	3	F	R1545	18-Dec-19	1382	1358	R1550 (M)(Fu)	0	1.9	3.22
54	2	F	Y1374	11-Mar-19	1118	1087	Y1376 (M) (Fu)	0	4.8	2.00
55	1	F	OY222	10-Sep-18	r112	781	OR200 (F)(HS)	Dam to R1497 (F)	1.5	1.70
56	3	M	R1550	18-Dec-19	1382	1358	R1545 (F)(Fu)	0	6.1	1.80
57	2	F	B1271	13-Feb-19	1149	1128	B1249 (M) (HS) B1273 (M) (Fu)	0	1.0	1.89
58	2	M	R1485	27-May-19	1160	1082	0	0	10.3	1.84
59	1	M	G1202	24-Aug-18	1069	1039	G1201 (M) (Fu)	0	6.0	1.84
60	1	M	OY232	17-Sep-18	r155	y76	0	Sire to R1519 (M) R1520 (M) R1497 (F)	2.0	3.09
61	2	M	G1423	08-Apr-19	1182	1143	G1418 (F) (Fu)	0	6.2	1.90

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
62	2	M	G1527	26-Aug-19	1183	1142	G1463 (F) (HD) G1413(F)(HS) G1415(M)(HS) G1410(F)(HS) G1411 (F)(HS)	0	9.3	2.03
63	2	F	G1241	06-Feb-19	1126	1092	G1227 (M) (HS) G1246 (M) (Fu) G1460 (M)(HS)	0	1.3	1.03
64	2	M	Y1376	11-Mar-19	1118	1087	Y1374 (F) (Fu)	0	13.1	1.70
65	2	M	B1306	27-Feb-19	1152	1127	B1231 (M)(HS) B1307 (M)(Fu)	0	7.9	1.94
66	1	F	G1135	09-Jul-18	1044	973	G1138 (Fu)	0	2.8	1.15
67		F	O1384	n/a	n/a	n/a	0		12.5	1.90
68		F	R1169	n/a	n/a	n/a	0		12.6	1.89
69	2	M	R1492	29-May-19	1077	1083	R1263 (M) (HS) R1489 (M) (Fu)	0	1.5	1.11
70	1	F	G1118	04-Jun-18	1024	932	G1122 (M)(Fu)	Dam to Y1374 (F) Y1376 (M)	3.9	1.53
71	X	M	B1074	29-Mar-18	946	924	B1072 (M)(Fu)	0	2.9	1.18
72	1	F	Y1077	16-May-18	873	819	0	Dam to R1489 (M) R1492 (M)	6.2	1.50
73	1	F	G1113	01-Jun-18	985	880	G1104 (M)(HS) G1105 (M)(HS)	0	1.6	0.83
74	3	F	B1539	25-Nov-19	1486	1337	B1544 (M)(Fu)	N	3.5	1.31

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
75	2	F	G1434	29-Apr-19	1221	1091	G1443 (F)(HS) G1444(F)(HS) G1438 (M) (Fu) G1436 (F) (Fu)	0	5.0	1.32
76	2	F	G1436	29-Apr-19	1221	1091	G1443 (F)(HS) G1444(F)(HS) G1438 (M) (Fu) G1434 (F) (Fu)	0	2.6	1.13
77	2	M	B1307	27-Feb-19	1152	1127	B1231 (M)(HS) B1306 (M)(Fu)	0	1.7	0.98
78	2	F	Y1473	17-May-19	Ob214	Ob194	Y1474 (F) (Fu) Y1476 (M)(Fu)	0	2.6	1.01
79	2	F	B1324	04-Mar-19	1166	1130	B1328 (F) (Fu)	0	3.5	1.33
80	2	M	B1503	12-Jul-19	Or200	B1186	0	0	3.0	1.28
81	2	F	G1410	08-Apr-19	1106	1142	G1413 (F) (Fu) G1415 (M)(Fu) G1411 (F)(Fu) G1527 (M)(HS)	0	7.5	1.57
82	3	M	B1533	18-Nov-19	1259	1249	0	0	3.3	1.14
83	2	F	B1328	04-Mar-19	1166	1130	B1324 (F) (Fu)	0	6.1	1.19
84	2	F	Y1474	17-May-19	Ob214	Ob194	Y1473 (F)(Fu) Y1476 (M)(Fu)	0	3.0	1.07
85	1	F	G1138	09-Jul-18	1044	973	G1135 (Fu)	Dam to Y1448 (M) Y1450 (M)	1.2	0.66
86	2	M	B1231	04-Feb-19	1147	1127	B1307 (M)(HS) B1306(M)(HS)	0	3.8	1.29

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
87	2	M	Y1379	20-Mar-19	1132	1078	Y1279(M)(HS) Y1383 (F)(Fu) G1379 (F)(Fu) Y1380 (M)(Fu) Y1381 (M) (Fu)	0	4.5	1.33
88	1	F	R1167	30-Jul-18	1018	992	0	0	2.7	2.47
89	1	M	B1223	17-Dec-18	1034	1027	B1183 (F) (HD) B1222 (M) (Fu) B1185 (M) (HD) B1187 (M) (HD)	0	4.5	1.22
90	2	M	G1438	29-Apr-19	1221	1091	G1443 (F)(HS) G1444(F)(HS) G1434 (F) (Fu) G1436 (F) (Fu)	0	2.0	1.03
91	2	M	Y1476	17-May-19	Ob214	Ob194	Y1473 (F)(Fu) Y1474 (F) (Fu)	0	2.9	1.01
92	1	M	B1128	11-Jun-18	937	986	0	Sire to B1249 (M) B1273 (M) B1271 (F)	1.2	1.03
93	2	F	O1393	29-Mar-19	Ob196	Oy179	O1399 (M)(Fu) G1512 (F) (HS)	0	2.5	1.01
94	3	F	G1512	19-Jul-19	1232	Oy179	O1393 (F)(HS) O1399 (M)(HS)	0	2.0	4.54
95	2	M	G1407	01-Apr-19	1205	1150	G1405 (M) (Fu)	0	2.6	3.15
96	1	M	B1187	06-Aug-18	1034	1009	B1183 (F) (Fu) B1223 (M) (HD) B1222 (M)(HD) B1185 (M) (Fu)	0	3.4	7.39

Sample no.	Gen	Sex	ID	DOB	Dam	Sire	Siblings Fu = full HD = Half Dam HS = Half Sire	Parent/ child	DNA yield ng/µL	DNA purity A_{260}/A_{280}
97	2	M	Y1279	13-Feb-19	1112	1078	Y1383 (F)(HS) G1379(F)(HS) Y1380(M)(HS) Y1381(M)(HS) Y1379(M)(HS)	Sire to Y1576 (F)	5.5	2.08
98	1	F	B1183	06-Aug-18	1034	1009	B1223 (M) (HD) B1222 (M) (HD) B1185 (M) (Fu) B1187 (M) (Fu)	Dam to G1527 (M) G1463 (F)	10.3	2.34
99	1	F	OB196	27-Jul-18	y97	w61	0	Dam to O1399 (M) O1393 (F)	1.7	8.28
100	1	M	Y1164	25-Jul-18	945	890	Y1161 (M)(Fu) Y1162 (M)(Fu)	0	3.3	2.45

8.2 APPENDIX 2: SAMPLE WILD-CAUGHT SPECIMENS (QUEENSLAND MUSEUM)

Lab ID	QM Tissue No	QM Voucher No	DNA Yield (Ng/µL)	DNA purity (A_{260}/A_{280})	Classification	Locality Name	Latitude (DMS)	Longitude (DMS)
W01	A003218	JM16932	1.2	1.58	Sminthopsis crassicaudata	Wallambilla district	26° 37' 38" South	149° 9' 51" East
W02	A003219	JM16933	85.8	1.81	Sminthopsis crassicaudata	Wallambilla district	26° 37' 38" South	149° 9' 51" East
W03	A006976	JM19742	36.2	1.88	Sminthopsis crassicaudata	Diamantina Lakes National Park	23° 33' 37" South	141° 7' 45.2" East