Outfoxing the fox: the use of single nucleotide polymorphisms to investigate population genetics of the invasive species the red fox and inform smarter fox control

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Declaration Statement

This thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the degree of International Bachelor of Science (Honours). It does not include any material published by another person without due reference within the text. The field and laboratory work presented in this thesis was performed by the author, expect where acknowledged. This thesis has not been submitted for a degree at any other University.

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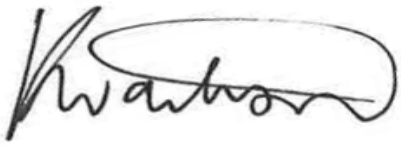
Signed: 

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Abstract

Invasive species have contributed significantly to the global biodiversity crisis. Since European settlement in Australia, it is estimated that invasive species have accounted for a significant portion of the decline and extinction of over 273 endemic terrestrial species. The introduction and spread of the red fox, *Vulpes vulpes,* to Australia has had pronounced damaging effects to native ecosystems across the island continent. As a globally significant predator and invasive species, the fox competes with native populations for resources and habitat and preys on small mammals and ground-nesting birds.

Population genetics can assist by providing information about the dynamics of invasive species that is beneficial for developing and improving effective control strategies. Through estimates of genetic diversity, population structuring, and genetic relatedness between individuals, information on the effectiveness of control strategies can be obtained, and recommendations to improve the efficacy of control programs may be possible. However, there are few recent population genetics studies of the red fox in Australia to date. This thesis aimed to add to the knowledge of the population genetic structure, diversity, and genetic relatedness of red foxes in eastern Australia through the analysis of a high number of biallelic genetic markers in a limited population of red foxes in the Illawarra and Shoalhaven regions.

Tissue samples were collected from foxes shot between March 2019 and March 2020 in the Illawarra and Shoalhaven regions. Extracted DNA from fox ear tissue samples were genotyped by Diversity Arrays Technology Canberra (Pty Ltd) using DArTseq technology. After quality control, a total of 17,898 biallelic markers were available for analysis for 93 individual fox samples. Genetic indicators were explored based on a one-population and a two-population model. For a one-population model, moderate values of gene diversities were found (H_E = 0.302), with moderate levels of inbreeding occurring across the genotyped foxes (F_{IS} = 0.0573). For a two-population model, moderate values of gene diversities were found

(*population 1 – H_E* = 0.311; *population 2 – H_E* = 0.309), with moderate values of inbreeding (*population 1 – F_{IS}* = 0.0412; *population 2 – F_{IS}* = 0.0566). Low population differentiation was observed assuming a two-population model (F_{ST} = 0.0176) with a large number of migrants per generation estimated (N*m* ~ 14). Interpretation of the genetic data in the context of the landscape may suggest that a major highway in this region could be used as a migration path. Additionally, this thesis observed high genetic relatedness among the sampled foxes, with eleven potential first-degree relative kinship groups present in the sampled foxes.

These results suggest the presence of a large panmictic population within the Illawarra and Shoalhaven regions, with high connectivity and free breeding occurring among the sampled foxes. By reducing gene flow across the landscape through targeting of contact zones for control, it may be possible to use genetic drift as a natural control agent through the reduction in alleles which are advantageous for invasive species dispersal and establishment. Given the limited number of foxes available and the limited area for the survey, it would be important to extend the current study. Additionally, focusing on possible migration corridors within this landscape for better control of fox populations should be explored.

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List of Abbreviations

Abbreviation	Stands for
H_E	Expected heterozygosity
H_O	Observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
IBD	Identity-by-descent
IBS	Identity-by-state
N	Number of individuals
Nm	Haploid number of migrants
SNP	Single nucleotide polymorphism

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1. Introduction

1.1 The threat of invasive species

Global biodiversity has been profoundly impacted upon by human activity through the facilitation of movement of introduced species (McGeoch *et al.* 2010). The introduction of non-indigenous species to areas beyond their native home ranges is accepted to be an historical consequence of human migration and trade (Clout and Russell 2007). These invasive species are considered a principal component in driving large-scale ecological change and represent one of the greatest threats to global biodiversity (IUCN 1999). This is due to their role in habitat degradation, destruction of natural biodiversity, and their contribution to ecosystem changes (Wilcove *et al.* 1998; Le Roux and Wieczorek 2009). The ecology of native species is often impacted by invasive species through increased competition for resources and increased levels of predation, therefore harnessing the potential to alter population dynamics and reduce the genetic diversity of native populations (Sakai *et al.* 2001), which is important for adapting to new and changing environments.

The decline and extinction of native species because of invasive species can have cascading effects across ecosystems. Altering of ecosystem processes, such as reduced disturbance of topsoil, has led to nutrient-poor soils in Australia with little organic matter accumulation and low rates of seed germination (Fleming *et al.* 2014). This is due to predation from invasive predators such as the feral cat (*Felis catus)* and the European red fox (*Vulpes vulpes),* where two-thirds of Australian digging mammals have become extinct or experienced high rates of population decline over the past 200 years (Fleming *et al.* 2014). Since European settlement in Australia, greater than 10 % of all terrestrial mammalian species have become extinct (Woinarski *et al.* 2015). Invasive species are currently recognised as the most important threat to native species in Australia, affecting 82 % (N = 1,257) of Australian threatened taxa

(Kearney *et al.* 2018). Phenotypic traits, or 'invasive characteristics,' are often linked to the ability of a species to successfully establish in a new environment and become invasive. Such traits include strong dispersal ability, abundant growth rate, elevated levels of competitiveness, and generalist versus specialist trophic interactions (Sakai *et al.* 2001). These characteristics can even differ between populations of the same species (Kolar and Lodge 2001). The ability to respond to selection and changes in genetic diversity is important for successful introduction and establishment to a new geographic region (Estoup and Guillemaud 2010).

Current management strategies of invasive species within Australia have proven to reduce local, small-scale population densities of animals, such as the feral cat (Denny and Dickman 2010) and the red fox (Saunders *et al.* 2010). However, the wide-ranging area covered by such animals and their dispersal abilities require a greater implementation of management strategies to see a change in the impact of invasive species on a larger scale. Therefore, understanding the spatial structure, dispersal and population genetics of invasive species is important to reduce or reverse the impact upon ecosystems. Specifically, molecular genetic techniques offer the opportunity to investigate patterns of dispersal (i.e. sex-biased dispersal), genetic diversity and patterns of gene flow which would otherwise be unknown using traditional survey methods. Using contemporary molecular techniques in conjunction with demographic data, these informed approaches can improve current understandings of population structure and dynamics of many invasive species (Hampton *et al.* 2004).

Current legislation is a limiting factor in further reducing the impact of invasive species in Australia, specifically for invasive species such as the red fox (McLeod and Saunders 2011). Fox control is not mandatory on private lands, specifically in NSW, where it has been shown that effective long-term pest control requires participation from a substantial number of landowners in an area (McLeod and Saunders 2011). As such, it is difficult to alter current management strategies for the benefit of reducing population densities of invasive species, such

as the red fox. Moreover, Australia has the highest mammalian extinction rate worldwide. Since European settlement, it has been estimated that approximately 273 native species have become extinct (Woinarski *et al.* 2015). As such, there is a great need to improve the understanding of the ever-changing ecology and biology of invasive species, such as the red fox, so that control efforts to manage and reduce the impact of invasive species can be informed. One way to do this is through the use of genetic techniques that enables the identification and monitoring of invasive species.

1.2 Molecular genetics and invasive species

Molecular genetic techniques have benefitted the discipline of ecology. Genetic techniques through the use of genetic markers have allowed for the investigation into gene flow between populations, population structure, and demographic changes that are difficult to physically observe (Morin *et al.* 2004; Helyar *et al.* 2011). Additionally, patterns of connectivity within and between populations, as well as genetic kinship analysis of populations, where individuals can be linked to a kin group rather than a specific population, can be informed by genetic techniques. Therefore, such information consequently allows for the design of management strategies specifically adjusted to the structuring of populations and their connectivity to other populations (Rollins *et al.* 2006; Estoup and Guillemaud 2010).

Molecular genetic techniques have been used to study invasive species. For example, molecular genetic approaches can be used to study the red fox which is a known nocturnal, and widely dispersing species, making it difficult to trap and study using mark-recapture techniques (Beltran *et al.* 1991). Similarly, radio-collars and camera-traps are also limited in the study of species such as the red fox. Such methods cannot inform the interaction between species and behavioural attributes (Cristescu *et al.* 2015). As such, genetic approaches offer the ability to study how these species interact, typically through reproduction, and an investigation into their

population dynamics, which would otherwise be unknown. Genetic investigation requires a sample from animals for analysis. However, while genetics requires an animal to be present in the environment for its genetics to be studied, genetic techniques need not always be invasive, and often do not require visual observation (Adams *et al.* 2019). For example, environmental DNA, which is DNA from scat, fur, offers the opportunity to investigate the population genetics of animals without observation. Therefore, non-invasive sampling techniques allow for the investigation of species that can be difficult to sample. Similarly, invasive techniques only require small amounts of tissue to sequence the DNA and therefore gain highly informative details about the genetics of the study species. Despite this, few published studies have investigated the population genetic structure of invasive species, such as the red fox, within Australia.

Through population genetic analysis, it is possible to assess the scale required for a management program implementation, as well as the effectiveness of current control strategies (Robertson and Gemmell 2004; Abdelkrim *et al.* 2005). Genetic monitoring can distinguish between surviving and reinvading individuals during or post-eradication campaigns (Abdelkrim *et al.* 2005). As such, this enhances the efficacy of eradication campaigns of invasive species and works toward reducing the economic cost of the campaign.

Analysis of the genetic structure of invasive species can assist in the development of effective and integrated management programs. For instance, genetic techniques can assist in invasive species management by predicting, and potentially preventing new invasions; by forecasting the efficacy of current control methods; by identifying whether the current scope of management is achievable through the identification of population units of manageable size and those which have a low re-introduction risk (Abdelkrim *et al.* 2005; Rollins *et al.* 2006). For example, management strategies of the invasive brown rat (*Rattus norvegicus*) have been informed by molecular genetic approaches. Using genetic analysis, estimates of gene flow and

genetic variation were studied for invasive rat populations. Robertson and Gemmell (2004) identified two genetically distinct and isolated populations of brown rats on a South Georgia Island, an island in the southern Atlantic Ocean, that could be extirpated without the risk of reintroduction. Similarly, management strategies of invasive starlings (*Sturnus vulgaris*) in Australia were informed using genetic approaches. Rollins *et al.* (2009) identified sex-biased female dispersal patterns and identified regions which should be more heavily targeted for control. This was based on the results of high rates of gene flow and emigration found from a source population (Rollins *et al.* 2009). Therefore, molecular genetic techniques hold the potential to effectively inform population management strategies of invasive species.

1.2.1 SNPs for population genetics studies

Genetics offers an innovative approach for the study of a species. Particularly, genetics offers an insightful approach to invasion biology studies and possesses the ability to inform the ecology and evolution of invasive and native species. The incorporation of population genetics into eradication and management efforts has been found to enhance the success of invasive species control, and assist in recognising possible positive outcomes of containment efforts (Abdelkrim *et al.* 2005; Rollins *et al.* 2006).

The most common polymorphism, which has more recently (within the past 10 to 20 years) replaced the use of traditional microsatellite markers are single nucleotide polymorphisms (SNPs). Single nucleotide polymorphisms, which are the substitution of a single base pair which occurs at a frequency of more than 1 % in a population (Morin *et al.* 2004), are recognised as the most common type of genomic variation (Figure 1). Other markers are less common, however, are considered highly informative genetic markers (Figure 1). Single nucleotide polymorphisms are biallelic, and as such do not provide the statistical power associated with traditional genetic markers. However, due to the high frequency of occurrence

throughout the genome, and the ease and high accuracy of parallel detection (Morin *et al.* 2004), SNPs are increasingly becoming the most widely used polymorphisms in genetic studies, from plants to microbes, to wildlife (Zheng *et al.* 2013; Vandepitte *et al.* 2014; Hendricks *et al.* 2017). Therefore, SNPs are increasingly becoming the marker of choice due to the associated abundance throughout a genome, resulting in a high statistical power.

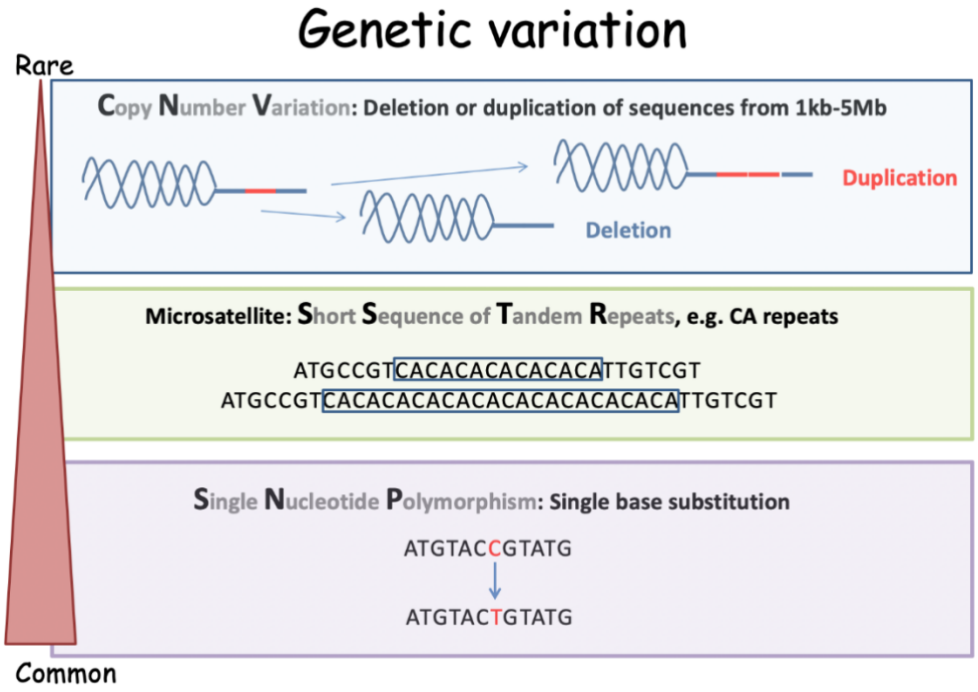

Figure 1: Characteristics of copy number variations (CNVs), microsatellites (SSTRs) and single nucleotide polymorphisms (SNPs) as forms of variation within the genome. Shown from the most common (SNPs) to rarest (CNVs) forms of genetic variation.

Today, genetic markers play a key role in estimating population genetic structure and are important in areas of research such as association mapping, evolutionary studies, forensics, and wildlife management (Carroll *et al.* 2018). Human genetic studies such as the Human Genome Project set the pace for using SNPs as the marker of choice for investigating phenotypes and disease (Guerra and Yu 2006). More recently, SNPs have been applied to investigating the population genetics of non-model organisms such as wildlife (Helyar *et al.* 2011). For example, SNPs have been used to study the genetic diversity and population

structure of the endangered Tasmanian devil (*Sarcophilus harrisii*). Hendricks *et al.* (2017) used 6,362 SNPs to estimate the potential spread of Devil Facial Tumour Disease across identified populations in Tasmania, and therefore inform conservation efforts to prevent a reduction in genetic diversity by introducing admixed individuals to isolated populations. Rick *et al.* (2019) studied the genetic diversity and admixture of the near-threatened burrowing bettong (*Bettongia lesueur)* using 21,267 SNPs. The study found it was advantageous to mix source populations in re-introduction efforts, specifically observing a two-fold increase in genetic diversity following hybridisation between two source populations. Moreover, SNPs have been used to sequence the genome of tame and aggressive red foxes (Kukekova *et al.* 2018). The study found that 103 loci were responsible for behaviour in foxes. As such, studies such as Kukekova *et al.* (2018), who were the first study to sequence the red fox genome two years ago, reveal the capacity for SNPs to be used to investigate complex parameters such as behaviour. As such, it is clear that SNPs provide a useful tool for the study, conservation and management of wildlife.

Effective and economical management of invasive species requires extensive knowledge of their demography, ecology, and impacts (Browett *et al.* 2020). Single nucleotide polymorphisms have widely been used in the estimation of genetic diversity and population dynamics of invasive species (Abdelkrim *et al.* 2005; Puckett *et al.* 2016; McCann *et al.* 2018). Similarly, SNPs can estimate the efficacy of targeting ecological corridors for invasive species control. An example for the latter has been used in dingo (*Canis lupus dingo*) populations which have been targeted by lethal baiting in five geographic regions of Australia. Cairns *et al.* (2018) used 58,000 SNPs and found high levels of inbreeding in targeted populations. They concluded that control strategies are working to reduce the genetic health of dingo populations whilst simultaneously reducing gene flow between populations. As such, the use of genetics in monitoring the control of invasive and pest species is an innovative technique that allows for

the evaluation of the efficacy of current control efforts. By implementing genetic techniques to the control of invasive species in Australia, such as the red fox, this may assist in the conservation of threatened Australian native species for which foxes pose a great threat.

1.3 Focal species: the invasive European red fox

The European red fox (hereafter 'fox'), *Vulpes vulpes* (order Carnivora, family Canidae), is the most widely distributed terrestrial mammal globally and is a significant invasive predator (Edwards *et al.* 2012). It has a natural range spanning the entire Holarctic, and an introduced range including Australia (Edwards *et al.* 2012). The ability for the fox to colonise biomes ranging from deserts to tundra represents a great ability to locally adapt to an environment, and aids in the species being a successful invader. The continued threat posed to native fauna by the red fox, particularly in continental Australia (Saunders *et al.* 2010; Woinarski *et al.* 2015) demonstrates the need for improved control strategies to reduce the pervasive threat red foxes pose (Kinnear *et al.* 2017; Hradsky *et al.* 2019).

1.3.1 Red fox biology

Foxes have a distinct appearance and are often described as a 'cat-like' canid. Foxes are medium-sized omnivores, with a long body and dense fur. Amongst the three colour morphs recognised globally, the red morph is the most common in Australia (Atlas of Living Australia n.d.). A range of morphological adaptations is thought to be linked to its hunting style for small mammals – including long hind limbs, light bones, and a small stomach (Short *et al.* 2002). Foxes display significant variation in size based on age, geography, and sex. On average, adults measure 35 to 50 cm high at the shoulder, with their weight ranging from 2.2 to 14 kg. Vixens (females) typically weigh 15 to 20 % less than the dog fox (male) (Atlas of Living Australia n.d.). Foxes have a generation time of one year and are sexually mature at approximately 10

months of age (Lariviere and Pasitschniak-Arts 1996). In Australia, breeding occurs between June to October. Female foxes commonly breed during their first autumn, however, in areas of high density, yearlings do not produce kits (offspring). In areas of lower density, 80 to 90 % of yearlings and 95 % of older vixens may successfully breed (Voigt and Macdonald 1984). Foxes mark their home ranges with urine, faeces, and use of scent glands. Reproductive status during the breeding season can be indicated by scent marking (Fawcett *et al.* 2012). The basic social unit of the fox is a monogamous pair; such social behaviour is the only example of cooperative behaviour exhibited by foxes (Lariviere and Pasitschniak-Arts 1996). Encounters between groups with large home ranges are rare and are aggressive, with agonistic behaviours rather than physical contact being the most common form of aggression (Lariviere and Pasitschniak-Arts 1996).

1.3.2 Red fox ecology and distribution

The red fox was first introduced to Victoria, Australia in the late 19th century for recreational hunting (Fairfax 2019). Foxes became established in Victoria, with a key prey species being the European rabbit (*Oryctolagus cuniculus)* (Fairfax 2019). Following their initial introduction, foxes appeared on the west coast of Australia (> 3,000 km) within half a century (Fairfax 2019). The spread of the fox throughout the Australian continent is also thought to be linked to the earlier introduction of their key prey species the European rabbit (Fairfax 2019). Current estimates suggest that foxes occupy approximately two-thirds of continental Australia. However, they are absent from far northern arid and tropical regions of Australia (West 2008). When seasonal conditions permit, the fox can penetrate the hot deserts of the interior as well.

As a solitary forager, the fox preys on rabbits, ground-nesting birds, and numerous small native mammals (Lariviere and Pasitschniak-Arts 1996). Due to the small size of the

prey, there is thought to be little cooperation between foxes for hunting and no defence of territory has been found by researchers (Dorning and Harris 2019). The red fox targets the critical weight range mammals weighing 35 g to 5.5 kg (Woinarski *et al.* 2015). Foxes can survive in small home ranges of 0.6 to 10 km^2 with high population densities of one to eight foxes per km^2 (Short *et al.* 2002). Social groups of foxes have been observed for cooperative breeding behaviours, with altricial and alloparenting behaviours exhibited by the breeding foxes, which are dominant in the social structure, and subordinates (Baker *et al.* 2004; Iossa 2008). The evolutionary stability of such social systems requires a high degree of relatedness between dominant and subordinate foxes, as such potentially allowing offspring to inhabit the same home ranges as parents (Baker *et al.* 2004). Foxes are thought to be monogamous during the breeding season, however, are known to exhibit polygamous behaviours when resource availability permits (Baker *et al.* 2004). Surviving in high population densities may mean that local movements for foraging and other activities occur up to 14 km from their home territory daily. This may make it difficult to control fox populations when their home ranges may differ significantly in size. Foxes will disperse from their mother typically within a year of birth and can disperse up to 80 km per year (Short *et al.* 2002). The ability for the red fox to disperse great distances and establish in a range of environments ensures its success as a pervasive invasive species and predator globally.

1.3.3 Red fox as an invasive species and predator

Invasive species, such as the fox, pose a great threat to native species. Listed as a 'key threatening process' under the *Environment Protection and Biodiversity* Act 1999, foxes threaten the biodiversity of native Australian species and are recognised as central to the high extinction rate of mammals exhibited across the Australian continent (Woinarski *et al.* 2015). The impact of foxes on native species can be inferred through the experimental design of native

species reintroduction studies. A study conducted by Short *et al.* (1992) revealed that in environments where foxes had been extirpated, there was an 82 % success rate for the reintroduction of six species of threatened wallabies, including the quokka (*Setonix brachyurus*) and brush-tailed rock-wallabies (*Petrogale penicillata*). This was compared to an 8 % success rate of reintroduction for environments where fox populations were not controlled. Augee *et al.* (1996) released hand-reared and relocated ringtail possums (*Pseudocheirus peregrinus*) into Ku-rin-gai national park near Sydney, Australia. Of the 118 individuals whose cause of death was determined, 52 % of individuals were killed by foxes, compared to 29 % killed by feral cats. More recently, Letnic *et al.* (2009) found that despite the removal of predation by dingoes through the installation of dingo-proof fencing, small to medium-sized mammal numbers continued to decline within experimental exclusion zones due to the presence of foxes within the fenced-off area. It is therefore evident that foxes play a key role in the decline of native Australian fauna and significantly contribute to biodiversity decline on the Australian continent.

Red foxes are recognised as a significant key threatening process that has historically and continues to contribute to the decline of Australian biodiversity (Woinarski *et al.* 2015). Historical observations of the red fox have documented the conspicuous decline of medium-sized marsupials at the time of the arrival of the red fox at a locality (Saunders *et al.* 2010). However, such observations do not rule out plausible alternative explanations, such as predation by feral cats or loss of habitat. Additionally, red foxes exhibit behaviours of surplus killing (Short *et al.* 2002), which is the killing of prey beyond the requirements of the energetic needs for foxes. Such a co-evolved predator-prey system is typically thought to have contributed to the rapid decrease in numbers of the Australian continent's endemic mammalian species (Short *et al.* 2002). Moreover, foxes compete with native species for habitat and resources and therefore threaten the presence of already declining native populations. Such has

been observed in the spotted-tail quoll (*Dasyurus maculatus)*, with a conservation status of near-threatened, where there is extensive overlap in the use of habitat and resource use (Glen and Dickman 2006). Field surveys have confirmed that spotted-tail quoll population densities are greatest in the absence of foxes or where fox sightings are rare (Saunders *et al.* 2010). As such, it is clear that foxes pose a serious threat to Australian native populations by competition for resources and habitat overlap. As such, there is a need to effectively control fox populations to reduce the impact upon native species.

1.3.4 Fox control strategies

Sodium fluoroacetate (compound 1080) baiting is the most common control technique used to manage fox populations in Australia (Saunders and Mcleod 2007; Saunders *et al.* 2010). It has the greatest success when baiting at large scales and high densities greater than five baits per km^2 (Hradsky *et al.* 2019). Foxes present in baited reserves have significantly higher mortality, shorter residency, and up to 80 % lower density than foxes in unbaited reserves (Marlow *et al.* 2015). Additionally, Thomson *et al.* (2000) conducted a large-scale baiting program with 45 radio-collared foxes. They found that more than 50 % of the collared foxes died within three days, resulting in an overall population reduction estimated to be greater than 95 %. This study highlights the importance of periodically baited buffer zones to minimise immigration to the central baited area. 1080 baiting specifically targets for canines, with a relative tolerance to 1080 bait of 1 for a lethal dose of 0.14 mg 1080/kg body weight. Native populations are less susceptible to baits. For example, possums have a relative tolerance to 1080 bait of 12, and eastern quolls have a relative tolerance to 1080 bait of 60 (Department of Primary Industries *et al.* 2017). However, as the bait specifically targets canines, domestic dog breeds are highly susceptible to baits. Therefore, on baited lands in the peri-urban landscape,

this can be deemed as a danger to pets. As such, a more informed approach is required to target fox populations and reduce their pervasiveness.

Other methods of control are thought to be less successful than baiting with 1080 poison. Hunting of foxes, including trapping, shooting, and dogging, are controversial due to perceived inhumaneness of the control measures (Saunders *et al.* 1995). The use of trapping is concerning because native wildlife may also be trapped and die, known as unintentional bycatch. Similarly, such methods have not proven beneficial in reducing fox population densities (Saunders and Mcleod 2007). The shooting of foxes is a popular technique, however, is time-consuming and not suitable in dense vegetation where foxes can take cover. As such, there is a clear need to increase the body of knowledge around the population dynamics, population structure and range of red foxes to inform control methods. Population genetics offers new and exciting tools to benefit the understanding of population dynamics of invasive species. Particularly, management and prevention strategies can be implemented based on information gained from genetic tools (Le Roux and Wieczorek 2009).

1.4 Genetics and the red fox

There is a large gap in the literature on the use of SNPs to estimate the population genetics of the red fox. A key study in the literature conducted using microsatellite genetic markers by Atterby *et al.* (2015) found that despite heavy culling of red fox populations throughout England, there was no evidence of substantial reductions to gene flow or lowered genetic diversity of populations. This was determined through estimation of admixture, high levels of immigration, and high levels of genetic diversity between and within red fox populations studied by these authors. Similarly, Norén *et al.* (2017) used 15 autosomal and one sex-specific microsatellite loci from 102 ($N = 102$) red foxes to determine population structure, estimate genetic diversity and dispersal rates. Norén *et al.* (2017) studied Scandinavian red

foxes, which threaten the persistence of the endangered Swedish Arctic Fox (*Vulpes lagopus).* The study found low population structure and high rates of immigration between tundra and adjacent boreal regions in alpine tundra biomes of Sweden. They determined that control measures should extend beyond the currently targeted regions of the Swedish tundra to adjacent boreal regions. It is therefore evident that control strategies can benefit from genetic analysis of fox populations to inform the efficacy of current control measures and provide guidance for future control strategies.

Genetics has also been studied more broadly in foxes, investigating genes associated with aggressive and tame behaviours in fox populations. A study conducted by Kukekova *et al.* (2018) identified genomic regions associated with tame and aggressive behaviours in foxes using SNPs. Domesticated, fur-farmed foxes selectively bred for over 50 generations for positive responses towards humans and foxes selectively bred for over 40 generations for aggressive responses towards humans were studied to identify 103 loci associated with behaviour in foxes. Notably, 13 of the identified genes were found to be the same genes associated in humans with bipolar disorder, another 13 genes were associated in humans with autism-spectrum disorder, and six genes were reported to be associated with aggressive behaviour in mice. While other genes out of the 103 associated genes had not been identified in human mental health and behavioural traits, some of these genes could be mapped to gene families relevant to these traits in humans and mice. To boost the information of the SNPs, haplotype analysis was performed; this looks at phased strings of SNPs in high linkage disequilibrium. Within one associated gene, *SorCS1* (Sortilin Related VPS10 Domain Containing Receptor 1), two haplotypes (*trq and lav*) were found to be rare in tame populations but frequent in aggressive populations; *olv* haplotypes were observed with a frequency of 60.6% in tame populations but were not observed in aggressive populations. This shows the importance of genetic variation in behavioural traits in foxes. In addition, this study is

particularly important in fox genetics literature, as it is the first time that the whole fox genome has been sequenced.

The use of genetic analysis has also been used to inform the understanding of invasion dynamics using a landscape-genetic approach in Californian red fox populations. A study by Sacks *et al.* (2016) used mitochondrial DNA sequences and 13 microsatellite markers to investigate source populations, connectivity and metapopulation dynamics of 402 (N = 402) red foxes removed in control programs in California. The study found estimates of high gene diversities for the entire sample (expected heterozygosity H_E = 0.55 to 0.71 across ten populations). Low connectivity was observed between the ten populations of red foxes recorded in this study. The low connectivity and restricted gene flow accounted for high population structuring consistent with origins from multiple introductions to California. The study observed two sites where immigration was occurring. This suggests the potential for recolonisation following eradication from a location. Therefore, the information attained from genetic analysis can be used to inform control strategies of the red fox in this area. This includes targeting of populations with low to no gene flow for eradication; and aiming to reduce the connectivity of immigrants between populations so that they then may be able to be targeted for eradication. This paper is one of the few studies relating to the use of genetics to inform management strategies of the red fox. As such, there is a clear need to investigate the population genetics of the fox to create a better understanding of the connectivity of fox populations in Australia, and therefore inform current management strategies used to target the fox.

2. Aims and Hypothesis

There is a need to use contemporary and innovative molecular genetics and population genetic techniques to inform and assist current fox control programs in decreasing the significant impact that red foxes pose to native species. Therefore, this thesis investigated the

genetic diversity of red foxes to improve fox control in south-eastern Australia. Using population genetic theory, this thesis investigated genetic diversity, gene flow and genetic relatedness of red fox populations in the Illawarra and Shoalhaven regions using SNPs.

The following hypotheses were tested:

H_1: genetic diversity will be high, with a lack of population structuring and high rates of expected gene flow across the landscape;

H_2: genetic relatedness will be low to moderate due to the moderate size of the study area, and high rates of dispersal across the landscape expected.

3. Materials and Methods

3.1 Study site

Fox ear tissue samples were collected from hunters contracted to the Berry to Budgong Fox Control Program (Shoalhaven Landcare Association Inc. 2019). The fox control program is funded by the NSW Government and is supported by Local Land Services and Landcare NSW. Post-mortem tissue samples were collected between March 2019 and March 2020. Tissue samples were placed in 96 % ethanol and stored at -20 °C. This study investigated the genetic diversity of foxes shot near the Bowral, Kangaroo Valley, Nowra, Mt Kembla, and Tomerong areas. Geographic coordinates (latitude and longitude) for all foxes collected were plotted using the ArcGIS Pro v2.4 (Figure 2). Additionally, basic data was provided by the hunter on fox biology, including estimated age, weight, sex, and an indication of health for the 94 foxes examined in this study (see Appendix 1). The indices provided were an estimate by the hunter and based on their professional opinion. The location of shot foxes covered a large geographic area of the Illawarra and Shoalhaven regions, ranging approximately 75 km from the furthest point of collection and covering an area of approximately 1,170 km^2 (Figure 2).

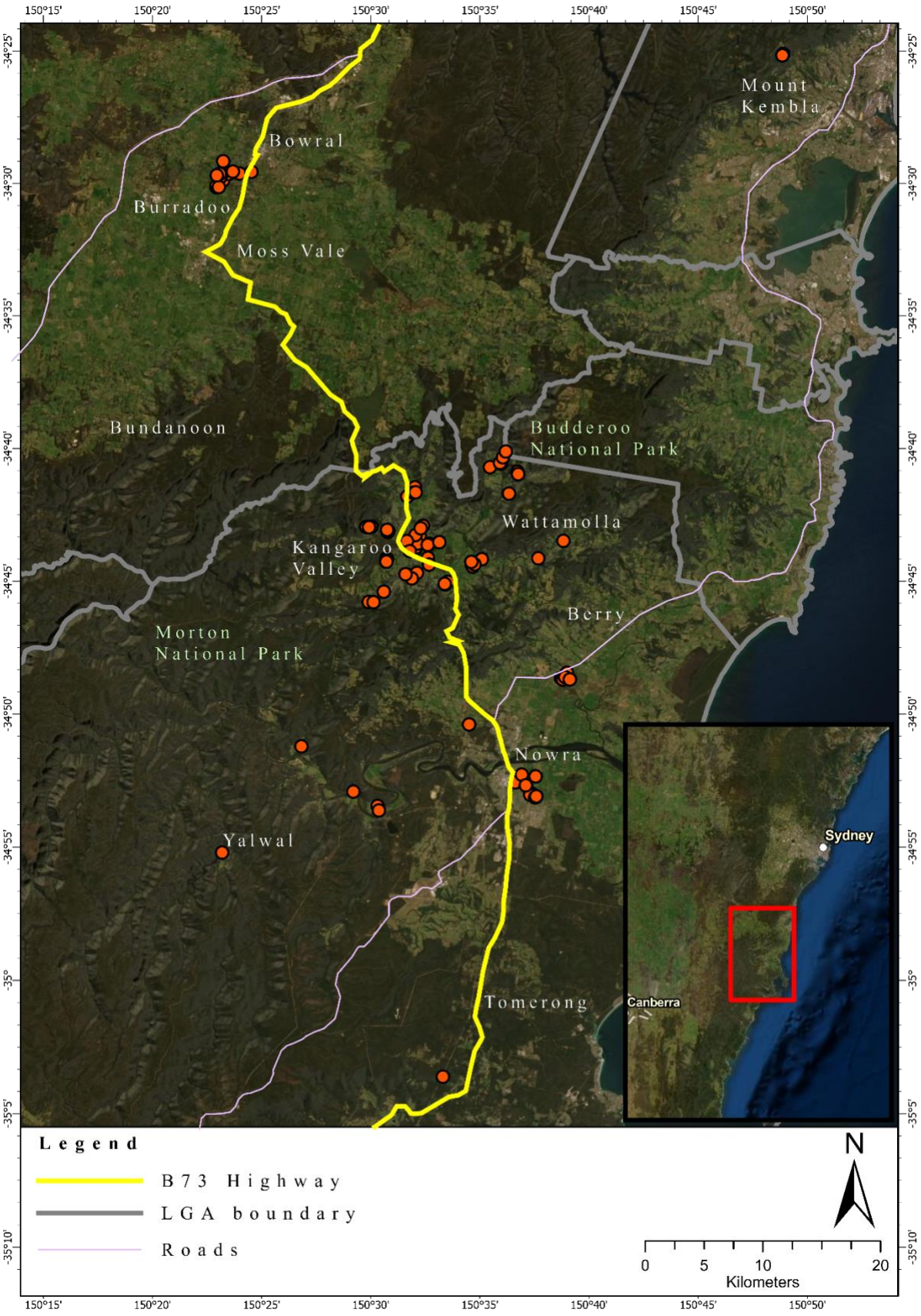

Figure 2. The geographic location of shot foxes. Orange points indicate the location of an individual fox. Each fox was legally shot by licensed hunters contracted by the Berry to Budgong Fox Control Program. Ear tissue was collected from foxes, stored in 96 % ethanol and frozen at -20 °C until used for further analysis. The map was created using ArcGIS Pro. LGA – Local Government Area.

The vegetation structure of sites near Bowral, Kangaroo Valley and Nowra consisted of cleared agricultural land that has highly fragmented remnant native vegetation. Mt Kembla consisted of many types of rainforest vegetation communities. Tomerong supports the growth of dry open low woodland through sandstone soils and taller forests through clay soils. Two major roads intersect the study area: the M1/A1 Princes Highway at Nowra (road travelling NE toward Berry); B73 Moss Vale Road through Kangaroo Valley (Figure 2). There are wildlife underpasses under the Princes Highway, forming part of the Berry bypass, that foxes are known to use (K. Mikac, unpublished data). The areas where most of the foxes were shot was mostly fragmented by clearing for agricultural land use. Approximately 10 foxes were shot in covered or forested sites near Mt Kembla, Yalwal, Nowra, Wattamolla, Tomerong, and Kangaroo Valley; two foxes were shot in the suburban area of Burradoo, NSW.

3.2 Ethics statement

Tissue samples were obtained from legally hunted foxes by licensed and contracted shooters by the Berry to Budgong Fox Control Program. This program has the aim of reducing the population density of foxes within the Illawarra and Shoalhaven regions, and therefore reducing the impact that foxes have on threatened native species. No foxes were killed specifically for this study. Therefore, in consultation with the University of Wollongong Animal Ethics Committee, no further ethics permissions were required to undertake the research.

3.3 Genomic DNA extraction

DNA was extracted from fox ear tissue (N = 94 foxes) using the QIAGEN DNeasy Blood and Tissue Kit protocol (QIAGEN, Hilden, Germany). A flow chart of the DNA

extraction process is provided (Figure 3). A piece of tissue approximately 0.5 cm^2 was excised from the fox sample provided. Cell lysis was induced by adding 20 µL proteinase K (600 mAU/ml) and 180 µL Tissue Lysis buffer provided in the DNA extraction kit. Samples were incubated overnight (approximately 12-14 hours) at 56 °C. Following incubation, 200 µL Lysis buffer and 200 µL 96 % ethanol were added to the lysate, then transferred to the supplied DNeasy mini spin column. The addition of the reagents before transferring to the spin column promotes selective binding to the column. The DNeasy mini spin column membrane combines the binding properties of a silica-based membrane with microspin technology. DNA binds to the membrane in the presence of high chaotropic salt concentrations. The buffer conditions enable specific adsorption of DNA to the silica membrane and optimise removal of contaminants and inhibitive enzymes. Two wash steps were performed in a microcentrifuge (Heraeus Biofuge Pico, Hanau, Germany) at 8,000 rpm and 14,000 rpm respectively using 500 µL wash buffer to remove cellular contaminants. Two final elution steps for DNA recovery were performed, using 200 µL of elution buffer each at 14,000 rpm for 1 minute.

3.4 Quality control

3.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using DNA extracted from five individual fox samples to confirm successful extraction of high molecular weight DNA and the quality of extracted DNA. Agarose gel electrophoresis separates DNA fragments by molecular weight when an electrical current is applied, with shorter fragments moving further toward the anode than longer fragments. A 1 % agarose gel was prepared; 1 g of agarose (Amresco, Ohio, USA), 4 mL 25X TAE Buffer (Ambion, Texas, USA), 33 mL GelRed® 3X Nucleic Acid Gel Stain (Biotium, San Francisco, USA), and 63 mL pure water. Reagents were brought to the boil to dissolve the agarose, cooled to 50 °C and poured into the gel mould with a comb to form

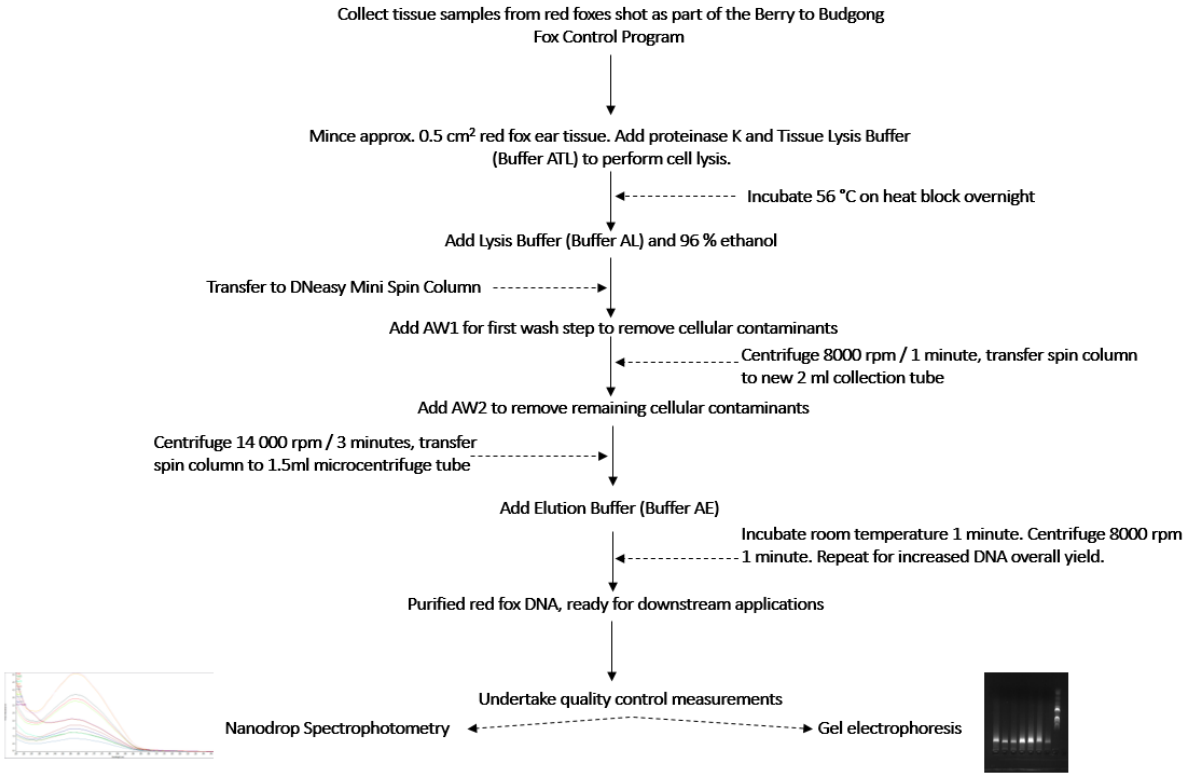

Figure 3. Flowchart illustrating the experimental procedure undertaken to attain extracted red fox DNA. The extraction process followed the protocol outlined by Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Fox tissue was lysed overnight on a 56°C heat block. Subsequent steps to extract the DNA from the tissue include precipitation of DNA with 96 % ethanol, two successive wash steps to remove cellular contaminants before elution from the Mini Spin Column. Nanodrop spectrophotometry and gel electrophoresis were undertaken as quality control measurements to ensure that the DNA extracted was suitable for downstream applications. Buffer ATL: Tissue Lysis Buffer; Buffer AL: Lysis Buffer; AW1: Wash Buffer 1; AW2: Wash Buffer 2; Buffer AE: Elution Buffer.

pockets where DNA was loaded for the electrophoresis step. The gel was allowed to settle for one hour at room temperature. Restriction enzyme digestion was performed using DNA from two individuals. The readily available restriction enzyme ApaLI (New England Biolabs, Ipswich, USA) was used to show that enzymatic downstream applications are not inhibited. For a 20 µL volume, 1 µL ApaLI (10 U/µL), 5 µL DNA, 2 µL of 10X Buffer Tango (Thermo Fisher Scientific, Massachusetts, USA) and 12 µL nuclease-free water were incubated at 37°C for 1.5 hours before loading on the gel.

Once the agarose gel had settled, the comb was carefully removed, and the agarose gel was added to a buffer tank containing 1X TAE buffer. All samples were prepared for loading to the wells in the agarose gel by adding 2 µL Gel Loading Dye, Blue (6X) (New England

Biolabs, Ipswich, USA). In addition to the samples, 5 µL of a molecular weight ladder (Quick-Load® Purple 100bp DNA Ladder, New England Biolabs, Ipswich, USA) was loaded to the wells. Electrophoresis was performed for 40 minutes at 70 V. The addition of GelRed® facilitated staining of DNA, as the dye interacts with the DNA to form a complex that fluoresces when presented under 302 nm wavelength of UV light. Separated DNA fragments on the agarose gel were visualised using UVP GelDoc-It Imaging System (Analytik Jena, California, USA). Multiple fragments of DNA were expected to appear on the gel for each sample due to the use of the whole fox genomic DNA for this study. The mean molecular weight of restriction enzyme digested DNA samples was expected to be smaller when compared to non-digested DNA.

3.4.2 Measurement of DNA concentration

Quality and concentration of extracted DNA samples were measured using a NanodropTM 2000 (Thermo Fisher Scientific, Massachusetts, USA). The optimal concentration for downstream applications as specified by Diversity Arrays Technology Pty Ltd (DArT, Canberra, Australia) was determined to be in the range of 50 to 100 µg/ml. Sample concentrations were standardised to approximately 75 µg/ml DNA. The total amount of DNA recovered for all samples was more than 15 µg, averaging 49 µg across the 100 extracted samples. $A_{260/280}$ measurements were taken to determine whether contamination with proteins was present within the extracted DNA samples and whether further purification was required for downstream applications. $A_{260/280}$ is an important quality control measure for DNA solutions. It is thought to reflect contamination with proteins, which have their maximum absorbance around 280 nm, whereas DNA has maximum absorbance around 260 nm. The ratio of $A_{260/280}$ should lie between 1.8 and 2.0. Lower ratios are indicative of protein contamination. Extracted DNA samples were within the $A_{260/280}$ range of 1.8 to 2.0 and averaged at 1.89.

3.5 Genotyping

Genotyping was undertaken by Diversity Array Technology Pty Ltd (DArT, Canberra, Australia) using the extracted fox DNA. DArTseq technology is informative for revealing genetic similarities amongst populations and species upon further analysis. DArTseq preferentially targets low-copy genomic regions over repetitive DNA fragments, increasing assay sensitivity and allowing for detection of a high number of informative SNPs across the genome (Melville *et al.* 2017). Next-generation sequencing (NGS) technology implemented in the DArTseq method uses the genomic representation of SNPs comprising of both constant and polymorphic fragments to reveal an informative DNA fragment. Illumina sequencing is the NGS technology of choice for DArTseq purposes. FASTQ files are generated and can be further used for filtering poor-quality sequences and SNP calls. Identical sequences are collapsed and analysed to correct low-quality bases. A reference genome is required for SNP marker alignment using BLAST (Ren *et al.* 2015). BLAST is also used in analytical pipelines to remove possible microbial contaminants.

The 94 samples were prepared on a 96-well plate, with the remaining two wells reserved for standards in the assays. DArT undertakes a complexity reduction method using methylation-sensitive enzymes that cut at exonic or near exonic regions (Sansaloni *et al.* 2010). These enzymes attach to the recognised cut sequence and pull out active areas in the genome, such as expressed genes. The complexity reduction of the whole genome allows for focusing more generally on important exonic regions i.e. those genetic regions with higher gene expression, and the removal of repetitive sequences which are often otherwise considered as junk. DNA polymorphisms, i.e. SNPs, are identified following alignment to a reference genome. The red fox genome assembly VulVul2.2 as available from NCBI (NCBI 2018) was used as a comparison sequence in this thesis.

3.6 Data analysis

3.6.1 Raw data processing

The SNP data received from DArT was loaded into the coding environment R v4.0.0 (R Core Team 2020) and converted into a genlight object, allowing the storage of multiple genotypes using the R packages adegenet v2.1.3 (Jombart and Ahmed 2011) and dartR v1.1.11 (Gruber *et al.* 2018). DartR is an R package used for loading SNP data provided through DArTseq methods. It provides the ability to apply filters based on locus metadata such as call rate, assigns individuals to populations, initial calculations of heterozygosity and package conversions.

3.6.2 SNP filtering

Descriptive statistics were analysed using the coding environment in R. Descriptive statistics are those which provide basic indices of genetic diversity across all individuals, such as degree of heterozygosity, or the number of alleles or polymorphic loci (Excoffier and Heckel 2006). The SNP dataset underwent a filtering process using R package dartR. The following filters were applied: A 90 % call rate filter for removing loci with a SNP call rate less than the provided threshold, i.e. SNPs which have 10 % missing genotypes or greater; the filter simultaneously filters for monomorphic loci; a filter for removing SNPs out of Hardy-Weinberg equilibrium with significance of $P > 0.05$; a filter for removing SNPs with very low minor allele frequencies (<1 %) due to low statistical power. Hardy-Weinberg equilibrium assumes (i) that large, (ii) randomly mating populations with (iii) no migration will remain in genetic equilibrium if (iv) no natural selection or (v) mutations are occurring within the population (Chen 2010). A $P > 0.05$ was returned if there is no significant genetic variation of a population, that is, the assumptions of HWE are met.

3.6.3 Population structure analysis and population assignment

In this thesis, two population models were assumed: a one-population model, and a model based on the most likely value of *K* populations determined by STRUCTURE and Structure Harvester analysis. Population structuring across the 94 individuals was tested for based on differences in allele frequencies across a range of assumed number of populations, *K*. The presence of distinct genetic differences amongst individuals indicates population structuring. This information was used to potentially estimate how distinct populations, if found, are interacting based on the location where they were shot. The program STRUCTURE v2.3.4 (Pritchard *et al.* 2000) was used to investigate the likelihood of individuals belonging to a putative population of origin (*K*). The program and method use Bayesian model-based algorithms for clustering genetic data (Pritchard *et al.* 2000). The program estimated allele frequencies in each cluster and population memberships for individuals. The proportion of genetic admixture within an individual, indicated by differences in allele frequencies, was used to estimate the presence of gene flow across populations. Admixture is the presence of genetic variation within an individual due to interbreeding between populations which have different allele frequencies (Skotte *et al.* 2013). Here, the admixture ancestry model with correlated allele frequencies was used. This model assumes a uniform prior, and without prior population information. The length of the burn-in period was set to 10,000 iterations, and the number of Markov Chain Monte Carlo repetitions was set to 1,000,000. The optimum number of clusters was determined by performing runs at K= 1 to 9 for 10 iterations. No greater number of *K* was deemed feasible due to the size of the study site, and foxes being wide-ranging and highly mobile species. The most probable value of *K* was determined using Structure Harvester v0.6.94 (Earl and VonHoldt 2012). The program uses the log probability of data [LnP(D)] and

delta *K* (ΔK) to determine the most probable value of *K* based on the rate of change in [LnP(D)] between successive *K* values.

Principal coordinate analysis (PCoA) was performed to determine genetic similarities and dissimilarities present within the dataset. This was calculated using R program dartR. The PCoA method uses Euclidean distance matrices to visualise patterns of genetic variations between individuals. If present, distinct genetic groups can be visualised using this method. It was expected that if there is large genetic differentiation between individuals, there will be distinct clusters of individuals present on the output plot. STRUCTURE allele frequencies and PCoA values were used to determine population assignment based on the most likely value of *K* populations.

3.6.4 Diversity analysis

The below statistics were calculated for each population model, respectively. The number of alleles was calculated using R package dartR. Observed heterozygosity (H_O) and genetic diversity per locus measured by expected heterozygosity (H_E) was estimated using R packages HIERFSTAT v0.04-22 (Goudet 2004) and dartR. Genetic diversity of a locus estimates genetic variation within populations and individuals. Values of heterozygosity ranges from 0 (no heterozygosity present) to near 1 (populations with a high amount of equally frequent alleles) (Harris and DeGiorgio 2017). Low values of H_E indicates severe effects of small populations, such as population bottlenecks or low rates of gene flow, and therefore the presence of inbreeding. High values of H_E indicates high levels of genetic diversity, with high rates of gene flow between populations. As foxes are highly mobile species, it was expected that genetic diversity indices will be high.

3.6.5 Genetic relatedness and population-based statistics

Pairwise genetic differentiation (F_{ST}) (Nei 1987), calculated using R packages HIERFSTAT and dartR, was used to test for differences in groups of individuals from the same area for the K >1 population model. The F_{ST} measures the amount of genetic variance explained by population structuring. A value of 0 indicates no differentiation between populations, while a value of 1 indicates complete differentiation. The haploid number of migrants (Nm) was estimated for the K >1 population model using R package HIERFSTAT. The Nm value indicates the movement of genes between populations, making them more genetically similar (Slatkin 1985). An Nm = 1 value indicates one individual exchanged between populations per generation time.

Genetic relatedness was estimated to conclude if current control strategies are working toward reducing the number of breeding individuals and therefore making the foxes more genetically related. PLINK v1.07 (Purcell *et al.* 2007) was used to estimate a matrix of pairwise identity-by-state (IBS) relatedness of foxes. The program computes an IBS similarity matrix of pairwise identities. The IBS relatedness matrix was plotted in a heatmap format using Clustvis v1.0 (Metsalu and Vilo 2015). A more yellow square indicates a value close to 0.7, which was the lower limit for this dataset. A value closer to one indicated the presence of highly genetically similar foxes. A value of one (white) indicated sample duplicates in the pairwise analysis.

PLINK was used to estimate identity-by-descent (IBD) of individuals, used to identify potentially related individuals. If two or more individuals share similar nucleotide sequences, which is known as identity-by-state (IBS), and in case the nucleotide sequence is inherited from a common ancestor, this is known as identity-by-descent (IBD) (Thompson 2013). Identity-by-descent indices are also useful for measuring changes in population structure, such as through population bottlenecks (Thompson 2013). The Z0 value refers to the proportion of no alleles

shared between the two individuals. The Z1 value refers to the proportion for which one allele is shared between the two individuals. The Z2 value refers to the proportion for which two (both) alleles are shared between the two individuals. A $\hat{\pi}$ value (which is a weighed value for Z1 and Z2 and as such reflects an estimate for the proportion of IBD) of approximately 0.5 indicates a potential first-degree relative. This was deemed to be a sibling or parent-offspring relationship. The type of relationship was estimated in consultation with the basic data available for the foxes. This included examining the approximate age range of the foxes, whether they were shot on the same day in the same location, and consultation with fox reproduction biology to determine if foxes were sexually mature at that. A Mendelian error test was performed to determine whether the parent-offspring relationships predicted was a likely condition. Potential relationships were subsequently removed in consultation with the number of Mendelian errors present. A greater number of Mendelian errors present indicates an unlikely relationship. A Mendelian error threshold around 400 errors (equals approximately 2 % error rate) was used to differentiate for relationships.

For investigating differences in allele frequencies between the two suggested populations as identified by STRUCTURE, dyads with high Z2 and high $\hat{\pi}$ values were removed. Since first-degree relatives share a greater proportion of their alleles, leaving both individuals in the samples would have otherwise introduced a non-acceptable bias to this analysis. Differences in allele frequencies between the two putative populations was tested for using PLINK.

The inbreeding coefficient F_{IS} was estimated using PLINK. Negative values of F_{IS} may indicate an excess of heterozygosity. Values close to zero may indicate a population in Hardy-Weinberg equilibrium. Values above zero indicate a degree of inbreeding between individuals, with higher values indicating greater levels of inbreeding (Wright 1965).

3.6.6 Sex-biased dispersal

Dispersal is the movement of individuals a distance away from their native home ranges. Sex-biased dispersal refers to the movement of one sex of a species more frequently away from their native home ranges. In mammalian species, sex-biased dispersal is a common phenomenon (Li and Kokko 2018). In this thesis, sex-biased dispersal was tested for using R package HIERFSTAT. The package tests for dispersal patterns based on sex and uses genotypes and rates of inbreeding to determine whether dispersal is sex-linked. The test was run using the mean allelic index count model based on the method presented by (Goudet *et al.* 2002) for 10,000 iterations. The test was performed for both the one-population model, and the two-population model. Significance was considered at a nominal value of $P < 0.05$.

4. Results

4.1 Genomic DNA extraction & quality control

DNA was successfully extracted from 94 foxes using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The total DNA recovered was more than 15 µg for all samples; a mean of 49 µg DNA was extracted across the 94 fox tissue samples. Extracted DNA samples were within the $A_{260/280}$ range of 1.8 to 2.0, with a mean of 1.89. The results of agarose gel electrophoresis (1 % agarose) using undigested DNA from foxes 1 through to 5 (lanes 1 through to 5 respectively) and restriction-enzyme digested DNA from foxes 1 and 2 (lanes 6 and 7 respectively) is provided (Figure 4). Lane 8 was a 100 base pairs (bp) DNA ladder, where the largest molecular weight observed in this ladder is 1,500 bp; the 'bright' bands corresponds to 1,000 and 500 bp, respectively. The digested DNA had a slightly lower molecular weight than the previous lanes, therefore confirming successful digestion of the extracted DNA and the ability to use this DNA for downstream applications.

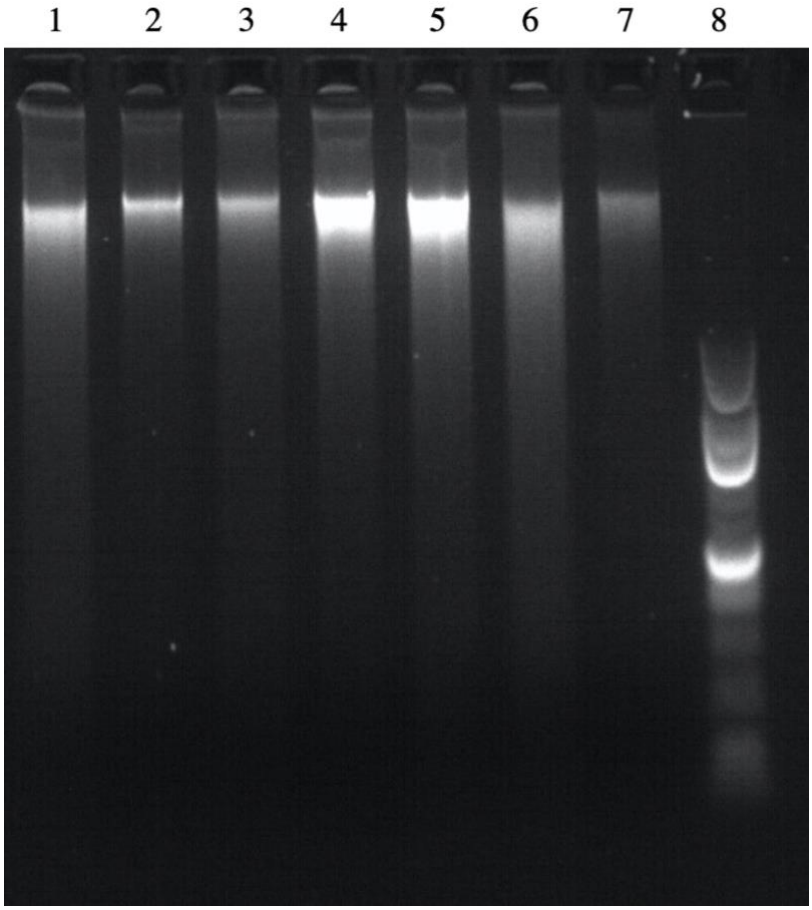

Figure 4. Extracted fox DNA and restriction enzyme digest of extracted fox DNA. DNA was extracted from fox tissue samples using protocols outlined by Qiagen DNeasy Blood and Tissue Kit. Extracted DNA was digested with ApaLI and incubated on a 56 °C heat block for 1.5 hours. Extracted and digested DNA was analysed by gel electrophoresis (1 % agarose) to confirm the presence of high molecular weight DNA, and to confirm integrity and digestibility for downstream applications. Lanes 1 to 5: DNA extracted from foxes 1 through to 5, respectively; Lanes 6 to 7: Extracted DNA digested with restriction enzyme ApaLI from foxes 1 (lane 6) and 2 (lane 7); Lane 8: Quick-Load® Purple 100 bp DNA Ladder.

4.2 Basic fox data

Basic data on the location of shot foxes, as well as general visual condition, sex, weight, and approximate age, was provided by the contracted fox hunters (Appendix 1). For 14 foxes, no data has been provided, therefore these foxes have not been included in the following graphs.

More male foxes (N = 51) were shot than female foxes (N = 29) (Figure 5a). Approximately 65 % of foxes were recorded to be in good condition (N = 51) (Figure 5b). Few

foxes were recorded to be in poor ($N = 3$) and fair ($N = 6$) conditions. Eighteen foxes ($N = 18$) were recorded to be in excellent condition. The largest number of foxes shot was less than one-year-old ($N = 28$) (Figure 5c). Fewer foxes were shot as the age range increased.

Most females were determined to be of good condition ($N = 22$) (Figure 6a). Few females were of both poor ($N = 2$) and fair ($N = 3$) conditions. Body condition was not provided for one female. Most males were recorded to be in good condition ($N = 29$). A large number of males was also recorded to be in excellent condition ($N = 18$). Most foxes less than one-year-old were of good condition ($N = 23$) (Figure 6b). No foxes above the one to two age range were of poor condition. Few foxes in the two to three ($N = 1$), three to four ($N = 2$) and four to five age ($N = 1$) range were of fair condition. Most of the foxes in the two to three age range were of good health ($N = 7$), followed by a portion of foxes in this age range considered to be in excellent condition ($N = 4$). Most of the foxes in the three to four age range were in excellent condition ($N = 7$).

4.3 Data analysis

4.3.1 Raw data processing

Using DArTseq technology, 33,735 SNPs were reported. Of the 94 fox DNA's sent for sequencing, 93 foxes were able to be sequenced. Fox 92 (Sex = female, age = <1 year) was excluded from further analysis due to sequencing failure. The following filters were subsequently applied for quality control measures of the data: a call rate filter set at a 90 % threshold removed 7,092 SNPs from the data set; a filter for removing SNPs out of Hardy-Weinberg equilibrium at an alpha value of $\alpha = 0.05$ and without Bonferroni correction for multiple testing removed 8,202 SNPs; a minor allele frequency filter for removing SNPs with frequencies <1 % removed a further 543 SNPs. Therefore, 17,898 SNPs were used for subsequent data analysis.

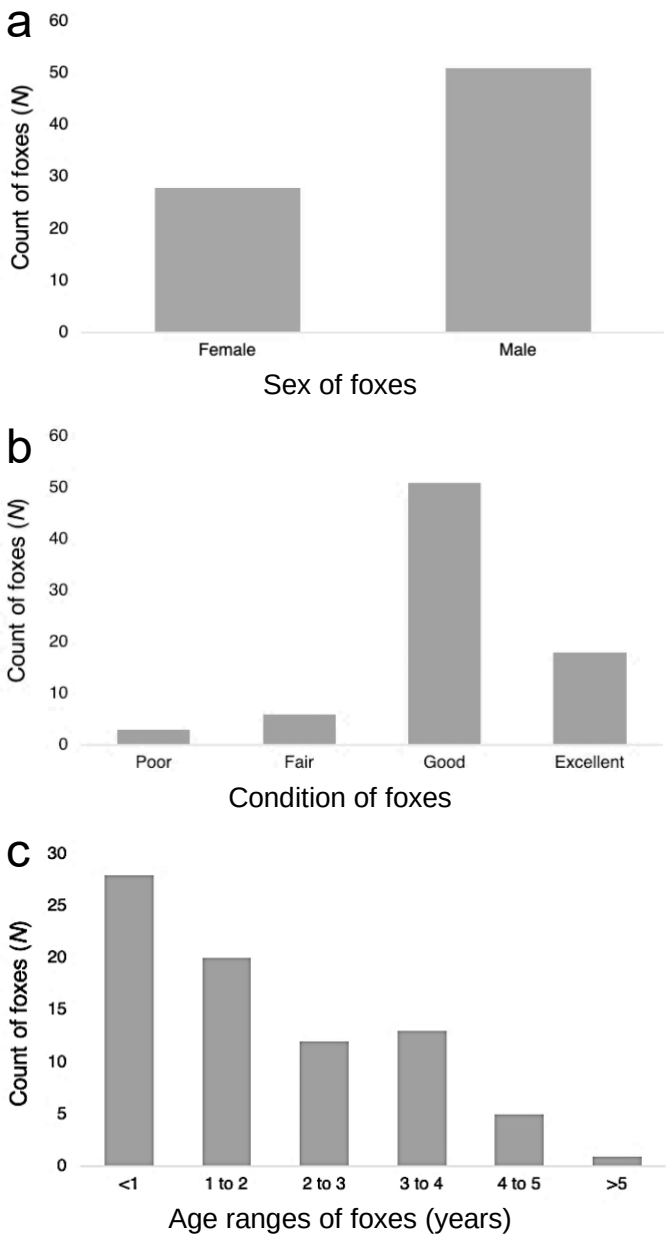

Figure 5. Graphs outlining results from the basic data provided by fox hunters. a) count of foxes by sex; b) count of foxes by body health condition; c) count of foxes by age.

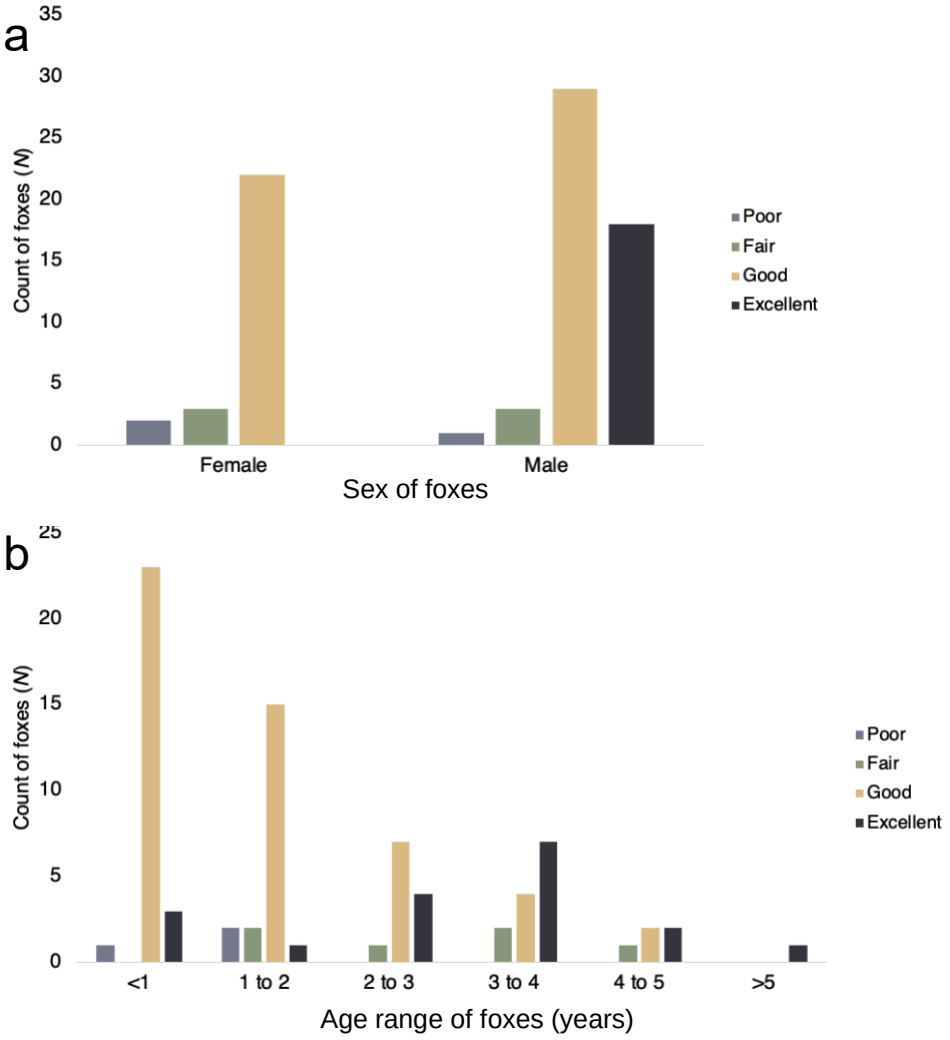

Figure 6. Graphs outlining the results obtained from the basic data provided by fox hunters. a) Body condition of foxes by recorded sex; b) Body condition of foxes by age range.

4.3.2 SNP informativeness

Minor allele frequencies (the frequency for the allele which occurs less frequently in the population), indicative of highly informative SNPs across the fox genome, were calculated using PLINK and visualised graphically (Figure 7a). Minor allele frequencies ranged from 0.012 to 0.5. The large majority (49.7 %) had frequencies between 0.15 to 0.4, indicative of good information value for genetic analysis. The proportion of missing SNPs missing per genotype was calculated using PLINK and visualised graphically (Figure 7b). The proportion of missing genotypes per locus ranged from 0 to 0.09 (0 % missing at a locus to 9 % missing at a locus). Complete genotyping was observed for 11,307 SNPs or 63 % of SNPs. This means, 100 % of the foxes had full genotype information available for 63 % of the SNPs. This left 6,591 SNPs, or 37 % of SNPs, with missing up to 10 % of genotypes for the foxes.

4.3.3 One-population model

For this model, one-population of foxes was assumed. Moderate observed heterozygosity (H_O) was observed across all foxes, with an estimated value of H_O = 0.280. Moderate genetic diversity, estimated by expected heterozygosity (H_E), was observed across all foxes, with an estimated value of H_E = 0.302. Moderate inbreeding was observed assuming the one-population model (F_{IS} = 0.0573 [-0.277 – 0.343]), with some high values of inbreeding observed for a small portion of foxes (F_{IS} >0.2, N = 6).

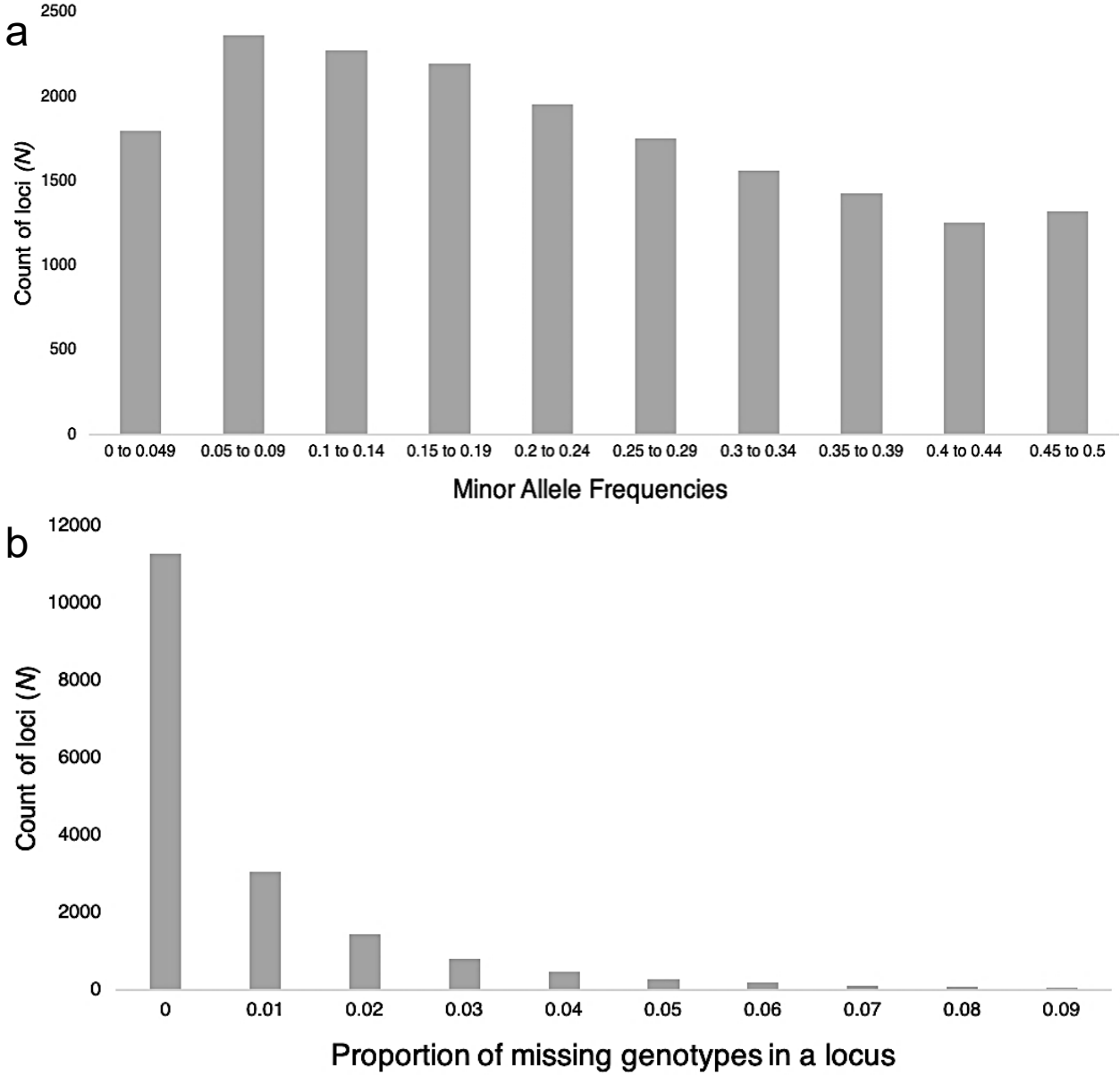

Figure 7. Summary graphs of informative SNP indices calculated using PLINK. a) Calculated minor allele frequencies ranges for the 17,898 SNPs. Minor allele frequencies ranged from 0.012 to 0.5. b) The frequency of missing genotypes observed per SNP. For 11,307 SNPs, complete genotyping of the 93 foxes was observed. The remaining 6,591 SNPs were not completely genotyped.

4.3.4 Fox population structure

STRUCTURE analysis revealed $K = 2$ was the most likely number of clusters or populations present within the sampled foxes. This was determined through execution of the Evanno method using Structure Harvester to determine the most likely value of K based on the rate of change of log probabilities of sequential K values. A sharp peak was observed at $K = 2$ (Figure 8). Foxes were assigned to one of two populations in consultation with results from STRUCTURE and principal co-ordinate analysis (PCoA) (Figure 9; Figure 10). The populations were arbitrarily named *population 1* and *population 2*, with correspondence to the colours on STRUCTURE and PCoA outputs. The STRUCTURE output (Figure 9) revealed 83 foxes whose allele frequencies correspond to one population at a defined level of 75%. Foxes which fell within the confidence margin were assigned to a population in correspondence with the PCoA output and values. The PCoA output (Figure 10) revealed two separate population clusters, with some admixture at the origin of the plot.

The map showing the location of shot foxes was updated to correspond to the population assignment for foxes (Figure 11). Red points on the map indicated assignment to *population 1*, where green points on the map indicated assignment to *population 2*. In the map, there was a cluster of *population 1* foxes located near Bowral, Mt Kembla, and Nowra. There was a large cluster of *population 2* foxes located around the Kangaroo Valley region. In this large cluster, there were individuals assigned to *population 1* also present.

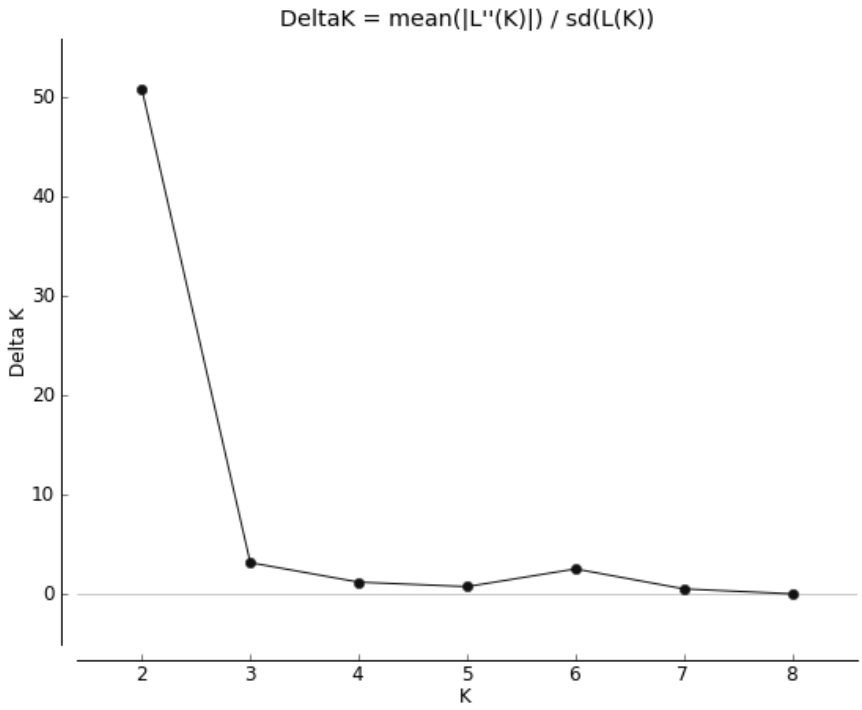

Figure 8. Results from Structure Harvester analysis to reveal the most likely value of *K* based on STRUCTURE results. The most likely value of *K* based on the rate of change of log probabilities of sequential *K* values. A sharp peak was observed at *K* =2. The value *K* = 2 is the most likely value of *K* >1 population model used in this study.

4.3.5 Association analysis

PLINK association analysis was used to broadly confirm that allele frequencies between two populations were significantly different. Given the large number of tests (17,898), the study-wide significant p-value was determined to be <0.0001. Association analysis of the putative populations revealed 19 of 17,898 SNPs were at a study-wide significance level, and therefore a statistically significant difference was found between allele frequencies for these 19 SNPs. Table 1 details the allele frequencies for *populations 1* and *2*, the minor allele based on allele frequencies, the polymorphism present at the SNP genotype. At a nominal p-value of <0.05, 3,350 SNPs were statistically significant.

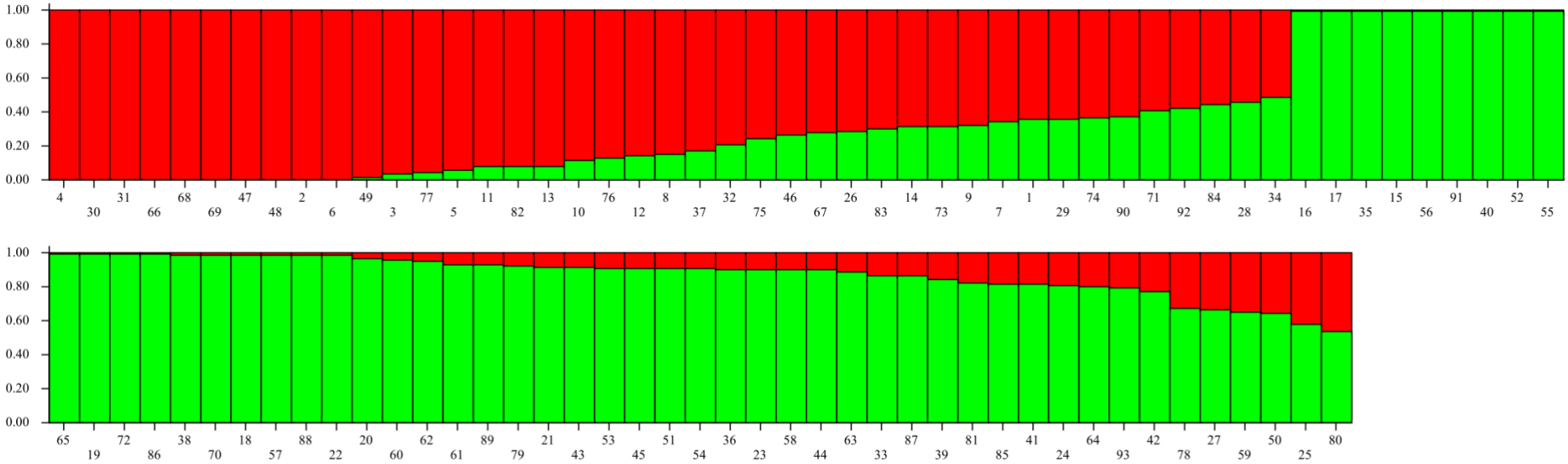

Figure 9. STRUCTURE bar plot for *K*=2 using a model based on admixture with correlated allele frequencies. Fox identities are represented by vertical bars. Distinct colours of the bars (red and green) represent the proportion of admixture (*Q*), or ancestry. Red corresponds to *population 1*. Green corresponds to *population 2*. Fox ID's are present on the X-axis and are ordered according to *Q* values presented on the Y-axis. N.B. the X-axis is continuous.

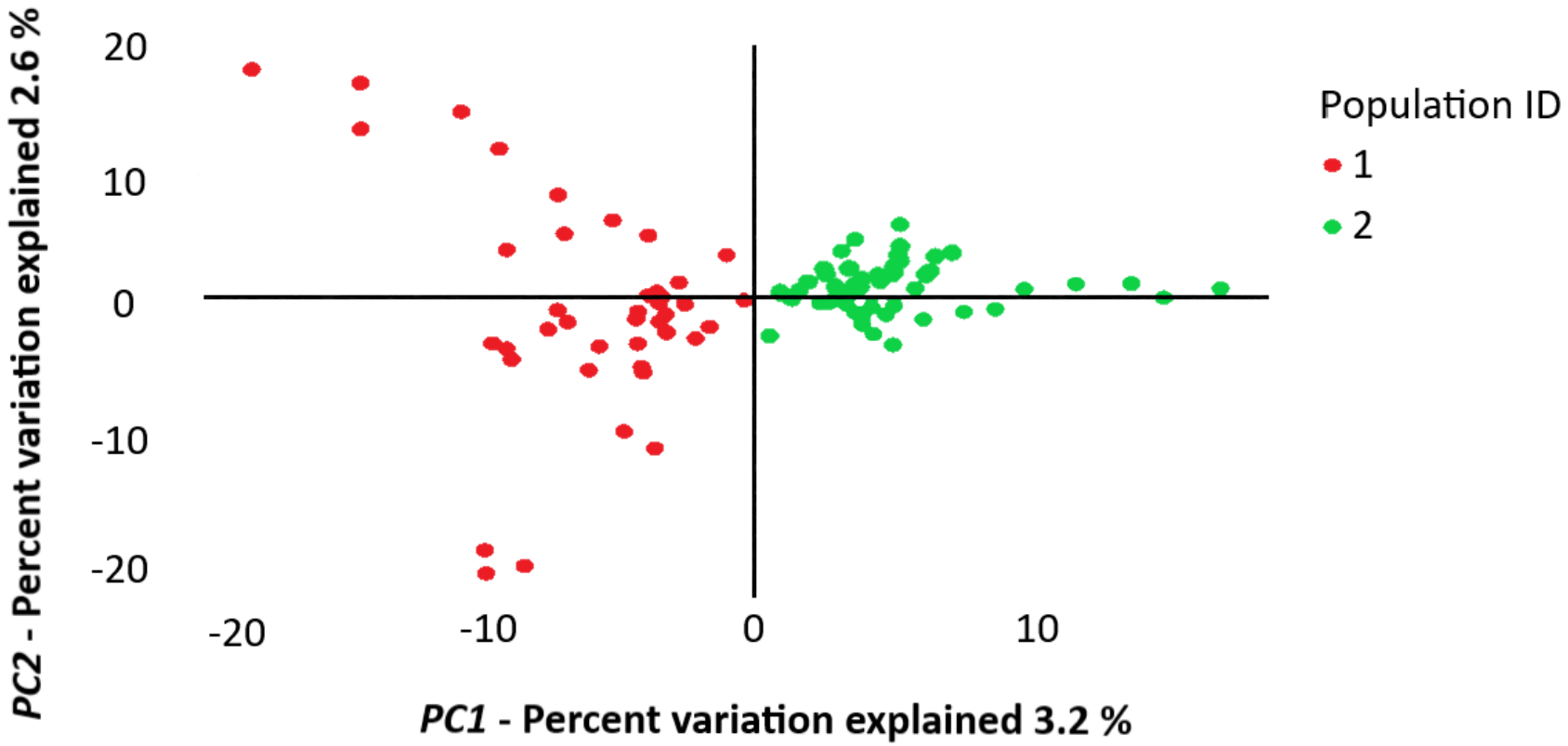

Figure 10. Principle Co-ordinate Analysis plot of genetic distances between two populations, *population 1* and *population 2*. PCoA between first and second axes covers 5.8 % of the variation in the data. Red points indicate membership to *population 1*. Green points indicate membership to *population 2*.

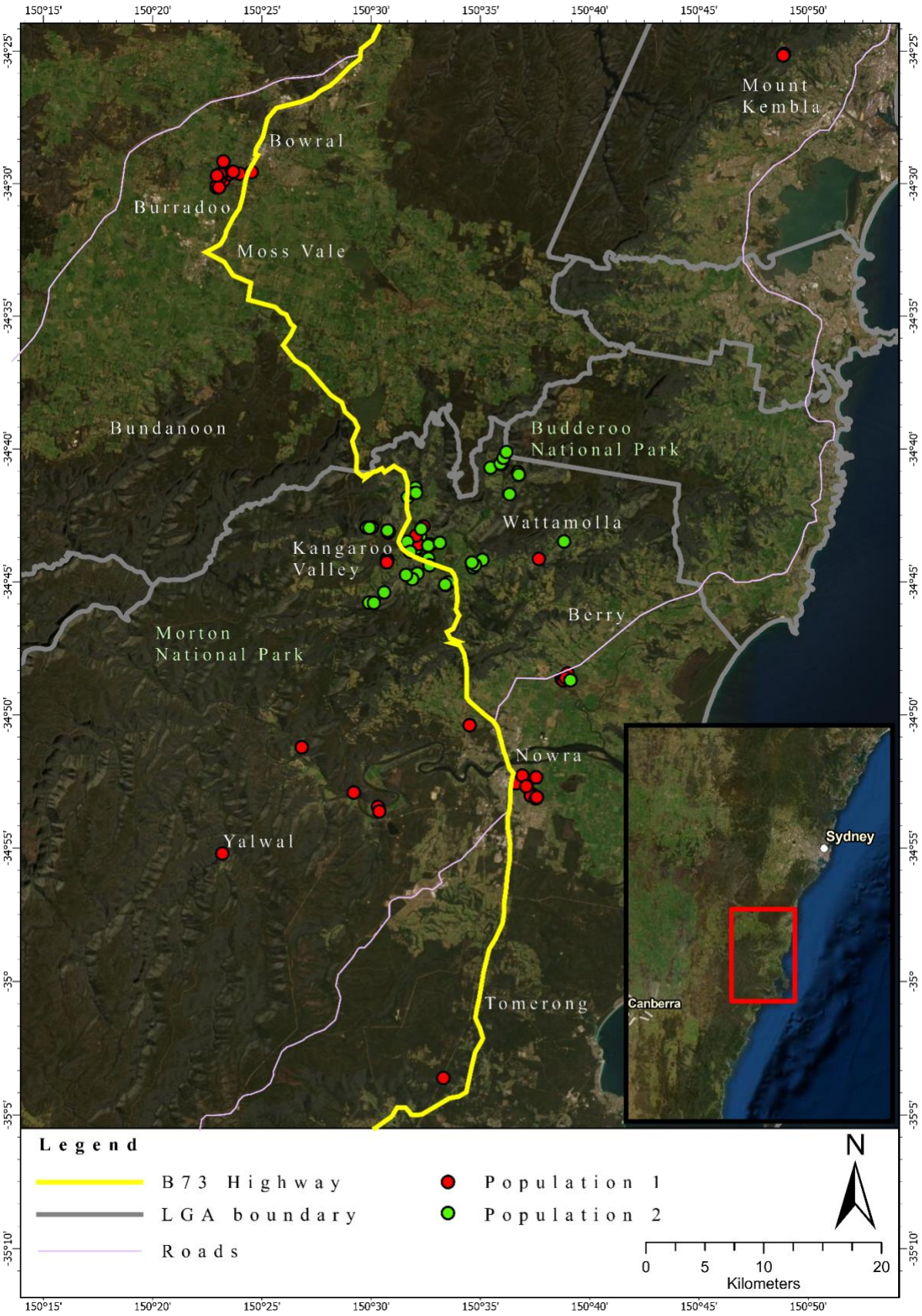

Figure 11. The geographic location of shot foxes. Red points indicate locations of shot foxes allocated to *population 1*. Green points indicate locations of shot foxes allocated to *population 2*. The yellow line indicates the B73 Highway. Grey lines indicate Local Government Area boundaries. Purple lines indicate other major roads in the area.

Table 1. Results from association analysis on PLINK. *SNP ID* refers to the SNP identifier supplied from DArTseq. *Polymorphism* refers to the alternate and SNP genotypes. *Minor allele* refers to the allele with the lower allele frequency in the total population. *Allele frequency – population 1* refers to the allele frequency for the genotypes of foxes in *population 1*. *Allele frequency – population 2* refers to the allele frequency for the genotypes of foxes in *population 2*. *p-value* refers to the significant p-value of the test with Bonferroni correction (P <0.0001). ***= P <0.0001

SNP ID	Polymorphism	Minor allele	Allele frequency – *population 1*	Allele frequency – *population 2*	p-value
10725763-16-G/C	G/C	C	0.3611	0	***
10723124-30-T/C	T/C	C	0.6111	0.125	***
100009517-51-T/G	T/G	G	0.7222	0.2024	***
57022061-8-C/T	C/T	C	0.4706	0.06818	***
10712442-5-C/A	C/A	A	0.2778	0	***
100016951-23-C/T	C/T	T	0.4444	0.05814	***
45537018-48-G/A	G/A	A	0.1111	0.6136	***
57022555-47-C/T	C/T	C	0.7222	0.2386	***
100027824-14-C/T	C/T	T	0.3056	0.01136	***
10715172-47-G/T	G/T	G	0.3056	0.01136	***
57020395-40-C/T	C/T	T	0.3611	0.03409	***
100022293-24-G/C	G/C	C	0.3333	0.02326	***
45541004-6-C/T	C/T	T	0.2941	0.01163	***
100035282-6-G/C	G/C	C	0.25	0	***
45544731-33-T/G	T/G	G	0.5	0.1047	***

45544731-33-T/G	T/G	G	0.5	0.1047	***
17675565-12-A/G	A/G	G	0.6944	0.2386	***
45533640-16-G/A	G/A	A	0.7222	0.2614	***
45536391-35-G/A	G/A	A	0.4444	0.07955	***

4.3.6 Two-population model

In this model, two fox populations were assumed. Foxes were assigned to one of two putative populations based on differences in allele frequencies from STRUCTURE results, as well as values from PCoA. Summary statistics were estimated for the two populations of foxes determined to be present within the samples (Table 2). *Population 1* was determined to have N = 42 foxes, and *population 2* was determined to have N = 51 foxes. Moderate observed heterozygosity was found for *population 1*, with an estimated value of H_O = 0.297 (± 0.147). Moderate expected heterozygosity for *population 1* was found, with an estimated value of H_E = 0.311 (± 0.146). Observed heterozygosity for *population 2* was determined to be H_O = 0.290 (± 0.148). Expected heterozygosity for *population 2* was determined to be H_E = 0.309 (± 0.147). Both H_O and H_E values for *population 1* and *population 2* were considered to display moderate gene diversities. Inbreeding coefficients (F_{IS}) for foxes in *population 1* indicates moderate levels of inbreeding. Values ranged from -0.283 to 0.300, with a mean F_{IS} value of 0.0412. Values of F_{IS} for foxes in *population 2* indicates moderate levels of inbreeding. Values ranged from -0.0178 to 0.193, with a mean F_{IS} value of 0.0566. The pairwise fixation index (F_{ST}) for *populations 1* and *2* was low and was estimated to be 0.0176. Pairwise migration rate Nm, indicating gene flow, was estimated to be approximately 14 individuals exchanged between the two populations per year.

Table 2. Summary table of statistics associated with fox population assignment. *Population* is the population ID; *N* refers to the number of foxes allocated to the respective population; H_O refers to the total mean of the estimated observed heterozygosity per population; H_E refers to the total mean of the estimated expected heterozygosity per population; F_{IS} is the estimated inbreeding coefficient per population; F_{ST} is the estimated pairwise *F* statistic estimated for the two populations. N*m* refers to the haploid number of migrants exchanged per generation.

Population	*N*	H_O (±SD)	H_E (±SD)	F_{IS}	F_{ST}	N*m*
Population 1	42	0.297 (±0.147)	0.311 (±0.146)	0.0412 (-0.283 to 0.300)	0.0176	13.955
Population 2	51	0.290 (±0.148)	0.309 (±0.147)	0.0566 (-0.0178 to 0.193)		

4.3.7 Genetic relatedness

The genetic distances were visualised in a heat map (Figure 12), with a colour key indicating the range of values present in the matrix. The pairwise relatedness values in the IBS matrix ranged from 0.7 to the highest value of 0.87 (Figure 12). The highest value obtained from the IBS relatedness was obtained between foxes 4 and 5 (IBS relatedness = 0.87). The values closer to 0.85 (yellow values – Figure 12) were those identified in further analysis as potential first-degree relatives. Small clusters of high values of genetic relatedness were observed (Figure 12), where many of these foxes were shot in the same geographic location or within the same time frame of each other. The mean IBS relatedness value present within the fox samples was approximately 0.74, indicating high genetic relatedness across the foxes sampled (Figure 12).

Identity-by-descent (IBD) analysis revealed potential familial relationships within putative fox populations (Table 3). The individual ID for the two potentially related individuals, as well as putative population assignments, are present in the first four columns (Table 3). No data on age or sex was provided for foxes 2, 4 or 5, therefore, no potential relationship could be concluded. All relationships were found to be within the assigned populations for the two-population model; there was no outbreeding observed amongst the populations. It was estimated that eleven first-degree relative kinship groups were present in the data set, with three individuals present in a kinship group for two potential kinship groups;

twenty-seven (29 %) of foxes were found to have a first-degree kinship with another fox sampled in this thesis. Five potential sibling relationships were found, estimated in conjunction with shot location and date, and age. One kinship group was thought to have siblings from different breeding seasons. Six potential parent-offspring relationships were found, estimated in conjunction with sampling location and date, and age.

A pedigree for potential fox families is provided for which the greatest support for parent-offspring relationships was found (Figure 13). Known fox individual ID's are identified within the pedigree; if an individual is unknown a "?" is present. Family A was thought to have a known father and known offspring, with an unknown mother (Table 3; Figure 13). A high $\hat{\pi}$ value ($\hat{\pi}$ = 0.4643) was obtained, with high allele sharing (Z1 = 0.4847; Z2 = 0.222). Family B was thought to have one parent and two offspring from different breeding seasons (Table 3; Figure 13). Individual fox 66 potentially fathered foxes 68 ($\hat{\pi}$ = 0.4673) and 69 ($\hat{\pi}$ = 0.443) in two consecutive breeding seasons. This was determined by the different age ranges for foxes 68 and 69. Foxes 68 and 69 were therefore thought to be half-siblings. Given the high $\hat{\pi}$ value between 68 and 69 ($\hat{\pi}$ = 0.5322), these foxes may have been mothered by a) the same mother, or b) two genetically similar mothers. Family C was thought to have both mating parents, and offspring (Table 3; Figure 13). A large portion of single allele sharing was observed for this potential kinship group (Z1 = 0.7882; Z1 = 0.8378), with $\hat{\pi}$ indicating a first-degree relationship between foxes 56 and 57 ($\hat{\pi}$ = 0.4269), and 56 and 58 ($\hat{\pi}$ = 0.4327) but no relationship present for 57 and 58 ($\hat{\pi}$ = 0). Family D was thought to have a known father and known offspring, with an unknown mother (Table 3; Figure 13). A high $\hat{\pi}$ value ($\hat{\pi}$ = 0.5239) was obtained, with high allele sharing (Z1 = 0.6691; Z2 = 0.1893).

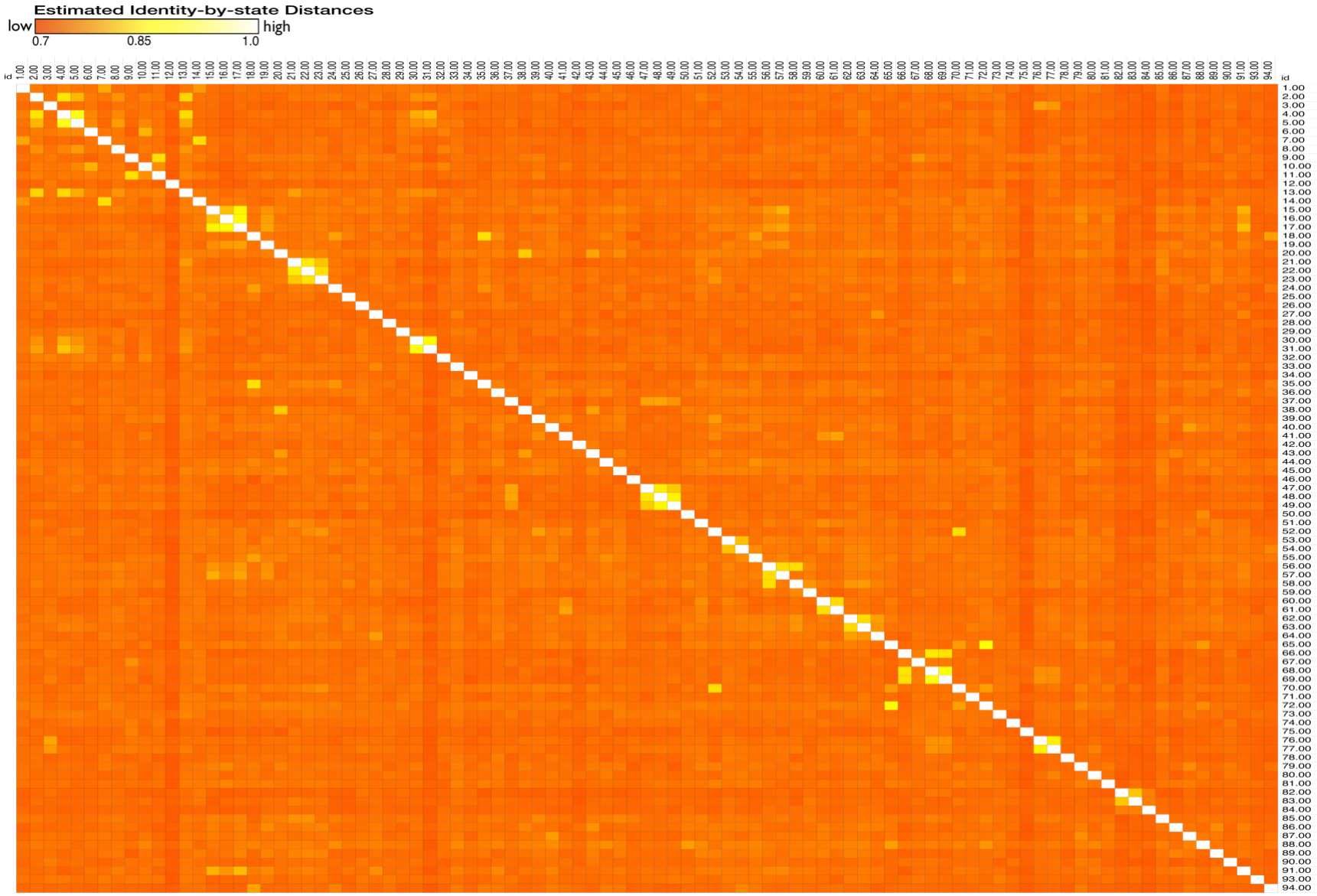

Figure 12. Pairwise genetic distance matrix of Identity-by-state distances generated by PLINK. Pairwise genetic distances calculated from 17,898 SNPS genotyped for 93 foxes. Pairwise genetic distances ranged from 0.70 to 0.87. The diagonal value of 1.0 represents a duplicated sample in the matrix. Fox identifications are present on the axes of the plot, from fox 1 to fox 94. Individual 92 was excluded from this study.

Table 3. Identity-by-descent (IBD) analysis based off genotype frequencies. Foxes are grouped together using estimates of pairwise IBD where the genotype frequencies between are more than is expected by chance in a random sample. No data was provided for foxes 2, 4, or 5, therefore, no relationship could be concluded. Z0 refers to the proportion of no alleles shared between the two foxes. Z1 refers to the proportion of one allele shared between the two foxes. Z2 refers to the proportion of both alleles shared between the two foxes. The $\hat{\pi}$ value refers to the proportion of IBD. A $\hat{\pi}$ value of approximately 0.5 indicates the possible presence of first-degree relatives.

Fox ID 1	Putative population assignment Fox 1	Fox ID 2	Putative population assignment Fox 2	Z0	Z1	Z2	π̂ value	Potential relationship
2	1	4	1	0.0754	0.8996	0.025	0.4748	-
4	1	5	1	0.4317	0.105	0.4633	0.5158	-
15	2	17	2	0.3059	0.4585	0.2356	0.4648	Potential siblings –15,16,17
16	2	17	2	0.0486	0.8324	0.119	0.5352	Potential sibling – 15,16,17
18	2	35	2	0.2798	0.6062	0.1141	0.4171	Potential siblings
21	2	22	2	0.2933	0.4847	0.222	0.4643	Parent-offspring
30	1	31	1	0.1415	0.6691	0.1893	0.5239	Parent-offspring
47	1	48	1	0.4201	0.3152	0.2647	0.4223	Potential siblings
48	1	49	1	0.371	0.3338	0.2951	0.4621	Potential siblings
52	2	70	2	0.1533	0.8165	0.0302	0.4385	Parent-offspring
56	2	57	2	0.1791	0.7882	0.0328	0.4269	Parent-offspring – 56 offspring of male 57
56	2	58	2	0.1484	0.8378	0.0138	0.4327	Parent offspring – 58 mated with 57 to produce offspring 56
65	2	72	2	0.3225	0.3633	0.3142	0.4959	Potential full siblings from different breeding seasons
66	1	68	1	0.141	0.7833	0.0757	0.4673	66 fathered 68
66	1	69	1	0.1807	0.7526	0.0667	0.443	66 fathered 69
68	1	69	1	0.2683	0.3989	0.3328	0.5322	Potential siblings
76	1	77	1	0.4133	0.2892	0.2975	0.4421	Potential siblings

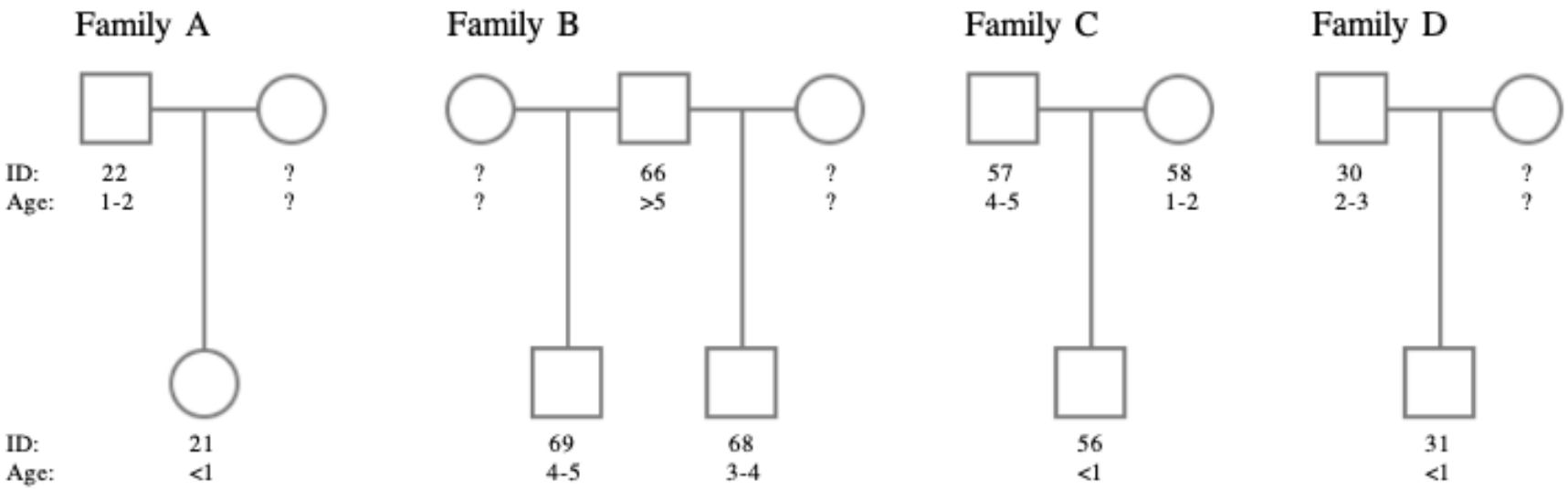

Figure 13. Potential family pedigree of sampled foxes using PLINK genome data analysis. Squares indicate a male; circles indicate a female. A "?" indicates that a maternal parent is unknown. Family A: Potential father fox 22 with unknown mother and male offspring fox 21. Family B: Potential father fox 66 with unknown mother and male offspring fox 68. Fox father 68 with unknown mother and offspring fox 69. Half-siblings fox 68 and 69 from different breeding seasons, as determined by their ages. Family C: Potential father fox 57 with potential mother 58 with male offspring 56. Family D: Potential father fox 30 with an unknown female with male offspring 31.

4.3.8 Sex-biased dispersal

Sex was not provided for 14 foxes; therefore, these foxes were excluded from this analysis. The test was run assuming both a one-population model and a two-population model. Significance was considered at a nominal value of $P < 0.05$. Sex-biased dispersal was found to be not significant ($P = 0.838$) assuming the one-population model and was found to be not significant for the two-population model ($P = 0.337$).

5. Discussion

5.1 Summary of results

This thesis investigated the genetic diversity and the presence of high levels of gene flow of the 93 foxes genotyped. It was hypothesised that genetic diversity of foxes would be high, and that gene flow between foxes would also be high. This study did not find significant evidence for high values of genetic diversity across either of the population models assumed. However, the values estimated were congruent with moderate genetic diversity across the foxes genotyped. This was true for both population models assumed. Additionally, high levels of gene flow were observed for the foxes genotyped assuming the two-population model. A value of approximately 14 individuals exchanged per generation, with a generation time of one year for foxes was found. Assuming the one-population model, it was evident that high levels of gene flow are apparent across the large spatial scale studied. Moreover, the findings of this thesis are consistent with high levels of genetic relatedness amongst the foxes sampled. This finding does not support the hypothesis of low genetic relatedness, and as such indicates that foxes in the Illawarra and Shoalhaven regions are homogenised genetically.

5.2 Heterozygosity and inbreeding

It was postulated that genetic diversity within fox populations would be high. However, this thesis found that genetic diversity, measured by H_E, was moderate (H_E = 0.302). This was not expected, as European fox populations have previously been observed to have high values of genetic diversity. Therefore, estimated values of heterozygosity observed in this study may represent the loss of genetic diversity when compared to fox populations in England, the fox country of origin (Fairfax 2019). A study conducted by Atterby *et al.* (2015) estimated H_E values ranging between 0.46 to 0.66 for nineteen fox populations across England. In this thesis, mean estimates of H_E was found to be 0.302. Similarly, moderate values of the inbreeding coefficient F_{IS} = -0.283 – 0.3 were estimated for the foxes. This indicates that this sample of foxes may have a reduced gene pool due to a high degree of genetic relatedness within the sample of foxes studied. This was also found in identity-by-state (IBS) analysis, where foxes had high values of pairwise IBS distances.

The reduced heterozygosity of the sampled foxes may also indicate the effects of range expansions of founder individuals first introduced to Australia. It is thought that eight successful releases of foxes in Australia in the 1860-70s established what would become one of Australia's most pervasive and threatening alien species (Fairfax 2019). The number of foxes released per event is unknown. The net result is that the introduction of foxes into Australia would have reduced genetic variation and increased genetic drift effects as the founders, and the surviving foxes following establishment would carry a smaller proportion of the total genetic variation of the source population from England. To determine whether the reduced gene pool is due to founder populations of foxes or due to the success of current fox control programs, future studies may wish to analyse DNA from historical samples of red foxes in Australia. Historical samples may include museum specimens, or DNA from foxes in the UK, which are ancestral populations of those in Australia. Additionally, the reduced

heterozygosity for this sample of foxes may indicate the effects of current fox control efforts. Through decreasing the gene pool available for foxes to breed freely, foxes may have become more related and therefore have low genetic diversity.

Reduced genetic diversity due to introduction away from native home ranges is common throughout invasion biology literature. This trend has been observed in species such as the common starling (*Sturnus vulgaris)* in Western Australia. A study conducted by Rollins *et al.* (2009) found the highest levels of genetic diversity to be present around sites of initial introduction. Genetic diversity was moderately reduced when starlings became established away from areas of initial introduction. This reduction in genetic diversity provides an example for founder effects of range expansion, where a small portion of genetic diversity is available for a newly established population. Similarly, invasive sugar gliders in Tasmania have been found to have reduced genetic diversity due to a severe genetic bottleneck which occurred when a small number of individuals were introduced to the island (Campbell *et al.* 2018). The information provided by these two studies can provide insight into the efficacy of current control efforts, as estimates of reduced genetic diversity due to range expansion can indicate whether populations continue to expand despite the control efforts in place.

5.3 Population assignment

It was hypothesised that gene flow (N*m*) between populations of foxes would be high. This is as foxes are a highly mobile and wide-ranging species. This was confirmed in this thesis, where an N*m* value of 13.955 indicates approximately 14 individuals are exchanged per population per generation. As fox generation time is equal to one year, it is therefore expected that 14 individuals are exchanged per year.

In this thesis, there was no conclusive evidence to support the assumption of the two-population model. The low value of the pairwise F_{ST} = 0.0176 estimated for the two fox

populations indicates a lack of genetic differentiation and structuring amongst the putative populations. This suggests that the allocation of two populations within the sample of foxes is not representative of two genetically distinct populations. Therefore, it is more likely that the one-population model represents the population structuring of the sampled foxes. As such, the results obtained in this thesis conclude that a single large panmictic population is present across the area sampled. This is also consistent with results from PLINK cluster analysis, where clustering of individuals revealed a lack of population separation. This may be due to a limitation associated with using Structure Harvester software. As the program relies on the rate of change between the log-likelihoods of [LnP(D)], it cannot accurately estimate for whether $K = 1$ is the most likely value of K. Therefore, a significant limitation lies within using Structure Harvester to accurately estimate the most likely value of K, particularly if there is no evidence of significant differences in fixation indices. Conversely, 19 of the 17,898 loci used in this study have statistical significance for different allele frequencies between the two populations, as determined by association analysis in PLINK. This information is in alignment with what is expected as the populations are in close geographic proximities. However, SNPs such as 10712442-5-C/A, 10725763-16-G/C and 100035282-6-G/C revealed that only *population 1* had alleles present for these loci, whereas *population 2* had no alleles present at these loci, assuming the two-population model (Table 1). These SNPs reveal highly statistically significant differences in allele frequencies. That one population has an allele frequency for the minor allele of 28%, 36% or 25%, respectively, and the other population does not show this allele at all (i.e. is monomorphic for this locus), is not expected by chance alone. Therefore, these putative populations may be becoming genetically distinct. This may be due to the result of intensive control of foxes in the Illawarra and Shoalhaven regions.

More generally, a lack of physical barriers limiting gene flow between clusters of foxes was found in this thesis. There does appear to be a high rate of gene flow present for foxes

across the whole Illawarra and Shoalhaven regions. The high values of gene flow found in the two-population model indicate a lack of landscape barriers acting to separate foxes geographically, therefore enabling for free breeding across the landscape. This is consistent with past fox literature, which suggests that foxes are a freely breeding, highly mobile species (Edwards *et al.* 2012). Figure 11 revealed the shooting locations of foxes, and showed foxes present from *population 1* and *population 2* in a cluster near Kangaroo valley, assuming the two-population model. Most foxes that were shot in the *population 2* cluster were shot alongside the B73 main road travelling between Nowra and Kangaroo Valley. This road also extends northwest to Bowral where a small cluster of *population 1* foxes are found, and south toward Nowra where a small cluster of *population 1* foxes are found. Therefore, foxes may be using the B73 as a movement corridor. This information is consistent with the findings of a study conducted by Towerton *et al.* (2011). A key aim of the study by Towerton *et al.* (2011) was to investigate the use of remote cameras to determine the effect of habitat and access tracks on detectability. They report that vehicular tracks were more frequently used by foxes than those off tracks. This may be due to roads providing fast access among foraging habitats (May and Norton 1996). Similarly, areas surrounding the road consists of both cleared agricultural land and remnant forests. Therefore, the use of a road could allow foxes a fast and easy path for foraging activities. However, it is also possible that the information gained is an example of sampling bias, where foxes were shot from vehicles and therefore, they are shot close to where the vehicles are travelling. Nevertheless, it would be beneficial for future studies to employ the use of radio-collared GPS tracking devices and wildlife cameras to determine the movement patterns of red foxes within the area. This would benefit knowledge about how the fox is using the landscape to travel within the region, and simultaneously allow for the targeting of known fox movement corridors for control.

5.4 Genetic relatedness analysis

It was hypothesised that foxes in this study would have low to moderate relatedness, which was not confirmed in this thesis. Foxes in this study were found to be highly related within the 93 successfully genotyped foxes. This was revealed through high values of IBS pairwise distances obtained by PLINK analysis with an overall mean IBS distance of approximately 0.74 found. Similarly, the moderate levels of inbreeding observed in this study (F_{IS} = -0.283 – 0.3) indicate the relatedness of foxes. The identity-by-descent analysis revealed 17 potential first-degree relative pairs within the sampled foxes, with 11 potential kinship groups. However, for some foxes, no basic data (i.e. age, sex) was provided. As such, potential relationship dynamics could not be determined for these kinship groups.

An interesting finding was observed through the IBD analysis for foxes 66, 68 and 69. All three foxes were males, with fox 66 potentially fathering fox 68 and 69 from different breeding seasons to different mothers. This is as foxes 68 and 69 were of different age ranges. The three male foxes were found to be shot in the same location on the same day. As per traditional fox biology, foxes are solitary and territorial canids (Dorning and Harris 2019). Iossa (2008) has found that males with a greater body mass have a greater home range size than those with smaller body masses. These three males (66, 68, and 69) were of the largest weights recorded across the sample of foxes, with potential paternal fox 66 weighing 7.8 kg, the largest weight recorded across the samples (Appendix 1). Similarly, two of these foxes (66 and 69) were recorded to be of excellent condition. As such, these foxes were believed to have minimal scarring present from recent fighting. Therefore, this provides some evidence for potential cooperation between the foxes rather than agonistic behaviours due to the close geographic proximities, which is not observed in fox biology. However, it is also possible that these foxes are forced to survive in close geographic proximities to each other due to the increasingly fragmented landscape that is becoming of the south coast region of NSW (Tozer

et al. 2010). Furthermore, no evidence of sex-biased dispersal was observed in this study. Although it is stated otherwise that male foxes will disperse greater distances from their birth den, the results of this thesis are not consistent with published literature (Robinson and Marks 2001; Walton *et al.* 2018). The abundance of resources present within the Illawarra and Shoalhaven regions available for foxes may discourage such dispersal and encourage philopatry (returning or stay in a particular area).

Invasive species may lose alleles which code for aggressive behaviours when the gene pool becomes reduced due to establishing in new environments (Tsutsui and Suarez 2003). This has been worked on in invasive insects, particularly invasive ants. For example, amicability between invasive, territorial ants has been well documented in the invasive insect literature. The term unicoloniality is used to describe the phenomenon in which ants from different, interconnected colonies behave amicably towards other nests in this 'supercolony'. This behaviour significantly contrasts with the territoriality that typifies many multicolonial ants (Tsutsui and Suarez 2003). The absence of costs associated with intraspecific aggression and territoriality are redirected toward efforts of colony growth, foraging, resource defence and competition (Holway 1998). It was found that genetic similarity may be used by the ants, and a recognition system may be established in which cooperative social behaviour is only displayed toward individuals who are deemed to be genetically similar (Reeve 1989). While it is known that foxes exhibit behaviours of cooperative breeding, it is unlikely that these three males were participating in this type of cooperative breeding social behaviour. This is as 'helpers' have been observed as subordinate females (Iossa 2008); there is no known documentation for subordinate male helpers. There is no other documented social cooperation in the fox literature. The information that has been published in invasive ant literature in conjunction with the uncharacteristic findings of this thesis therefore provides a platform for future research into fox social behaviour within Australia. Particularly, research into the

relationship of kin and behaviours of related foxes to observe if a disjunct from known fox biology is occurring.

5.5 Informing fox control

The presence of one large panmictic population of foxes was found in this thesis. Based on the results of this thesis, there are reservations about the use of current broad-scale population management strategies to reduce the population densities of red foxes effectively and sustainably across south-coast NSW. It may be more feasible to employ strategies more closely aligned to the minimisation of economic impact, rather than population reduction methods (Hulme 2006). In addition to the impact on native species, there is a heavily debated impact on the agricultural industry (Saunders *et al.* 2010). Estimates from 2004 suggest that the economic impact of red foxes is over AUD $227 million annually (McLeod 2004). Asset protection may therefore prove a more effective strategy than broad-scale culling and population reduction methods. This may include dutifully increasing 1080 baiting densities around lambing events to prevent asset loss. Conversely, studies have shown that foxes will predate regardless of the presence and magnitude of baits (Greentree *et al.* 2000; Gentle *et al.* 2007). However, a study conducted by Greentree *et al.* (2000) found that periodically baiting for foxes three times a year reduced the minimum percentage of lamb carcasses identified as killed by foxes from 1.5 % with no control to 0.25 % with baiting events three times a year. Therefore, to reduce the impact of the red fox on agricultural assets, it is important to ensure that landholders participate in periodical baiting programs. Similarly, using population density estimate techniques, such as using camera-traps and remote cameras, is important to estimate bait densities required for control (Rowcliffe *et al.* 2008). Understanding the population densities of foxes is important, as low bait densities result in low encounter rates, and high bait densities may encourage caching of baits (Towerton *et al.* 2016); as such, resulting in low

mortality. Through estimation of population densities and subsequent genetic monitoring, the impact of control programs can be determined by changes in population dynamics and genetic diversity, and therefore inform whether control strategies need to be reformed.

Landholders should recognise that immigration from an unbaited property is highly likely despite the magnitude of baits laid (Gentle *et al.* 2007), therefore, emphasis should be invariably placed on periodical baiting and the cooperation of landholders to maximise asset protection. To reduce hesitations surrounding unintentional poisoning of domestic pets, it is also suggested that the implementation of a metal 'collar' surrounding baits is trialled. The metal collar surrounding a bait injector is designed so that only fox snouts can fit inside to grab the bait head and pull it out to inject a bait; the fox has a slim, long snout that this collar has been specifically designed for (Sleath and Price 2019). The results of this study have not been published; however, initial findings have shown that dingoes cannot access the internal bait. Therefore, this may be a useful bait adaptation that specifically targets for foxes and reduces the susceptibility of domestic pets and other native animals to baiting.

Effective management of foxes should consider how foxes make use of the landscape to disperse. In particular, the movement corridors being exploited for dispersal purposes should be investigated and targeted for fox control in the NSW south-coast region. By targeting movement corridors which aid in dispersal, this may reduce gene flow and decrease the dispersal range of foxes. Such has been suggested for invasive starlings in Western Australia (Rollins *et al.* 2009). Furthermore, as a large-scale population was identified across the entire Illawarra and Shoalhaven regions, focused control efforts should aim to have a knockdown of population densities. However, reinvasion will be rapid, therefore making long-term population reduction efforts difficult. This has been found by Thomson *et al.* (2000), where a 95 % population reduction of foxes was estimated. Reinvasion of the baited area occurred in the following autumn when juveniles began to disperse from their natal dens. The study,

therefore, suggested that baiting regimes also take into consideration periodical baiting during the juvenile dispersal phase. Such regimes may prove more cost-effective forms of fox management, as this may reduce the overall number of baiting campaigns required each year (Towerton *et al.* 2016).

This thesis has found that foxes may be using the B73 to travel between Nowra and Bowral through Kangaroo Valley and to access agricultural land present adjacent to the motorway. Therefore, periodical baiting along the motorway and adjacent areas may reduce connectivity between groups of foxes and subsequently reduce gene flow across the landscape. A push toward reduction of gene flow, the structuring of populations across the landscape, and allowing genetic drift to assist in control may be beneficial for fox control. This is through the potential loss of advantageous alleles which allows foxes to be successful invasive predators and therefore leaving these foxes susceptible to selection. Similarly, implementing genetic monitoring of the invasive species should be a preliminary step in the management process to assess the effectiveness of control efforts (Abdelkrim *et al.* 2005; Rollins *et al.* 2006). Through ongoing observations of reduction in genetic diversity and increases in genetic relatedness, this may indicate the success of control strategies. Similarly, the implementation of a genetic monitoring program can investigate source populations if reinvasion to an area occurs (Abdelkrim *et al.* 2005), and therefore if gene flow is occurring from source fox populations. As such, monitoring can inform where to specifically target to reduce gene flow across landscapes.

The estimates of reduced genetic diversity and high levels of inbreeding found in this thesis indicates that current fox control measures are working toward reducing the genetic health of fox populations. This thesis is limited as it samples a limited geographic area. As foxes are wide-ranging and highly mobile mammals, this thesis would have benefitted from sampling foxes across a larger spatial scale, potentially allowing for greater genetic diversity

to be obtained due to geographic distance. Future studies may wish to implement a broader-scale investigation into how the population genetic dynamics of red foxes are or are not altered throughout the state of NSW. This may include a continent-wide investigation into the patterns of genetic diversity across Australia, and how genetic diversity differs from where foxes were first released in southern Victoria to their northernmost distribution. This may inform whether control efforts have assisted in the reduction of genetic diversity, or if the moderate levels of genetic diversity found for the population investigated in this thesis are due to founder effects of range expansions. From comparing genetic diversity, this thesis may be able to conclude on function, i.e. genetic variants, which may be important for certain behaviours which may help foxes to survive in various geographic regions. The removal of genes in 'tame' farmed red fox populations (those which do not display aggressive behaviours toward humans) from 'aggressive' red fox populations (those which display aggressive behaviours toward humans) has been observed in a study conducted by Kukekova *et al.* (2018). Therefore, it may be reasonable to speculate that red foxes in Australia may display this loss of aggressive genes due to population bottlenecks, genetic drift, and prolific dispersal in Australia in a relatively short time period.

The low F_{ST} and high migration rates found for the two-population model suggests that these areas should be wholly managed for control efforts. This includes a focus on control management strategies around the Kangaroo Valley region, where a movement corridor for the panmictic population along the B73 motorway was found. These contact zones should be concentrated on for population density reduction as admixture between the genetic groups can increase the adaptive potentiation of invasive species, and therefore enhance their invasion success further (Fraser *et al.* 2013; Sacks *et al.* 2016). Similarly, the results suggest that high gene flow is apparent among areas separated by 1,170 km^2 encompassing the study area under investigation in this thesis. As aforementioned, targeting contact zones and reducing

connectivity across the landscape could assist in promoting genetic drift and further reducing the heterozygosity of red foxes. Therefore, the removal of advantageous genetic variations that are 'pre-adapted' to conditions within the local environment may be promoted (Le Roux and Wieczorek 2009); and therefore, allow for the natural genetic control of foxes.

Population reduction and asset protection continue to be the most practical strategies for management of red foxes. The results indicate that there is not sufficient isolation within the red fox population for eradication within the Illawarra and Shoalhaven regions without sufficient risk for reintroduction to the region. The way forward for long-term fox management would be to promote the reduction of gene flow across the region and to strive for the genetic isolation of foxes. The use of natural genetic control to promote genetic drift and allow selective pressures through the reduction of advantageous alleles may allow for the natural reduction of fox population densities. If fox populations can be genetically isolated enough with minimal gene flow occurring by dispersal, this may allow for better control of foxes and a shift to genetically structured populations (Robertson and Gemmell 2004).

5.6 Future Research

Through genetic analysis of foxes present within the Illawarra and Shoalhaven regions, rates of migration (N*m*), values of genetic relatedness between individuals, and the values of genetic diversity present in foxes across the region have been identified. This investigation is vital to current fox management strategies, as it indicates the range of connectivity of foxes across the region. However, the assessment conducted in this study is a preliminary investigation into the movement patterns and genetic diversity of red fox populations within south-eastern Australia. Upon the conclusion of this study, it was determined that there are additional areas of research required to understand the movement patterns of the red fox and therefore inform fox management strategies.

Further studies investigating the population genetics of the red fox would benefit from a greater spatial scale of sampled foxes. This would provide a greater understanding of how related foxes from a greater area are, and therefore inform where (or if) connectivity reduces with distance. This may allow for targeting of contact zones which reduce gene flow across landscapes. Similarly, it would be beneficial to determine potential ecological corridors through techniques such as camera-trapping of areas that are known to be frequented by fox prey. This could determine the density of foxes in that area and therefore provide information to inform the number of baits required for successful control efforts. Also, it would be beneficial to study fox movement using tracking devices (i.e. GPS collars) to determine frequented movement corridors. As such, targeting of these movement corridors and ongoing genetic monitoring may provide insight into whether gene flow is reduced and therefore whether control efforts are specifically targeting to reduce the connectivity of foxes.

6. Conclusion

This thesis is the first study that has investigated the population genetics of the red fox on a landscape scale in Australia. The results indicate the presence of a large panmictic fox population spanning across the Illawarra and Shoalhaven regions. This suggests boundless connectivity for foxes across the landscape, and as such indicating that current control efforts are not working to reduce the dispersal ability and the connectivity across the landscape. Particularly, the potential use of the B73 as a movement corridor allowing for the increased connectivity of foxes across the landscape. These results have implications for informing future control of foxes across the region, where contact zones should be targeted to structure populations and therefore reduce gene flow across the region.

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Appendices

Appendix 1: Basic data that was provided with shot foxes, including coordinates where shot, approximate age, sex, weight, and condition of health.

Variable	Description
Individual	The number assigned to the individual for further analysis based on the chronological order that the fox was shot by the hunter.
Date	The date in DD/MM/YY format that the fox was shot.
Latitude	Latitudinal coordinates that the fox was shot in degrees.
Longitude	Longitudinal coordinates that the fox was shot in degrees.
Age (years)	The approximate age in years of the shot fox as estimated by the licensed hunter - opinion of the hunter based on age biology of the fox, distinguishing features based on age. Not provided for 14 foxes.
Sex	The sex of the shot fox - Male or Female. Not provided for 14 foxes.
Weight (kg)	The approximate weight of the shot fox in kilograms (kg). Not provided for 14 foxes.
Condition	The visual condition of health that the shot fox was found in - opinion of the hunter based off presence of mange, excessive scarring due to fighting, size of the fox. Not provided for 14 foxes.

Individual	Date	Latitude	Longitude	Weight (kg)	Age range (years)	Sex	Condition
1	13/3/19	-34.4861	150.3874	-	-	-	-
2	13/3/19	-34.49425	150.3905	-	-	-	-
3	13/3/19	-34.49805	150.3873	-	-	-	-
4	13/3/19	-34.49399	150.39164	-	-	-	-
5	13/3/19	-34.49386	150.39983	-	-	-	-
6	1/5/19	-34.5026	150.3823	-	-	-	-
7	10/4/19	-34.4948	150.3835	-	-	-	-
8	10/4/19	-34.4926	150.3948	-	-	-	-
9	1/5/19	-34.5016	150.3820	-	-	-	-

Individual	Date	Latitude	Longitude	Weight (kg)	Age range (years)	Sex	Condition
10	1/5/19	-34.4960	150.3853	-	-	-	-
11	1/5/19	-34.5024	150.3840	-	-	-	-
12	1/5/19	-34.4928	150.4090	-	-	-	-
13	1/5/19	-34.4940	150.3848	-	-	-	-
14	1/5/19	-34.4950	150.3825	-	-	-	-
15	13/2/19	-34.715785759	150.500489752	3.2	<1	Female	Good
16	13/2/19	-34.715958893	150.496630691	3.5	<1	Male	Good
17	13/2/19	-34.719232436	150.512963361	3	<1	Female	Poor
18	13/2/19	-34.718488354	150.513607968	5	2 to 3	Male	Excellent
19	13/2/19	-34.756547514	150.510009314	4	<1	Female	-
20	15/2/19	-34.696793598	150.527977524	4.4	2 to 3	Female	Good
21	15/2/19	-34.691024941	150.533802714	2.1	<1	Female	Good
22	15/2/19	-34.694413699	150.532425116	3.6	1 to 2	Male	Excellent
23	15/2/19	-34.694134817	150.534234315	6.1	2 to 3	Male	Excellent
24	19/2/19	-34.735818913	150.512040333	3.6	1 to 2	Female	Good
25	19/2/19	-34.721513995	150.537395499	5.8	2 to 3	Male	Good
26	25/2/19	-34.839770162	150.575043027	6.2	2 to 3	Male	Excellent
27	2/3/19	-34.695051931	150.605503027	5.5	2 to 3	Female	Good
28	8/3/19	-35.060248839	150.555129795	4.5	1 to 2	Male	Good
29	8/3/19	-34.810695990	150.645171468	7.1	4 to 5	Male	Good
30	8/3/19	-34.807431500	150.649778435	6.5	2 to 3	Male	Good
31	8/3/19	-34.811650250	150.646499685	2.5	<1	Male	Good
32	8/3/19	-34.810182120	150.648432387	5	2 to 3	Female	Fair
33	10/3/19	-34.724637610	150.647026137	4.5	1 to 2	Female	Good
34	10/3/19	-34.735562430	150.627900497	5.1	3 to 4	Male	Fair
35	10/3/19	-34.739410854	150.577041464	3.5	1 to 2	Female	Good

Individual	Date	Latitude	Longitude	Weight (kg)	Age range (years)	Sex	Condition
36	4/5/19	-34.811567249	150.652000072	6.1	3 to 4	Male	Excellent
37	5/5/19	-34.418635680	150.814940020	3.8	1 to 2	Male	Poor
38	7/5/19	-34.746362713	150.530902177	2.6	1 to 2	Female	Poor
39	7/5/19	-34.736185747	150.584867249	7	4 to 5	Male	Excellent
40	7/5/19	-34.740752446	150.577865027	4.5	1 to 2	Male	Good
41	7/5/19	-34.739471218	150.579169247	3.5	1 to 2	Female	Good
42	7/5/19	-34.737881038	150.576725771	6	3 to 4	Male	Good
43	7/5/19	-34.744757205	150.535307583	4.2	1 to 2	Female	Fair
44	7/5/19	-34.748229281	150.531370844	6.8	3 to 4	Male	Excellent
45	16/5/19	-34.745615494	150.526512174	6.3	3 to 4	Male	Excellent
46	25/9/19	-34.853583221	150.447051376	5.1	1 to 2	Male	Good
47	29/10/19	-34.419417243	150.814277788	1	<1	Male	Good
48	29/10/19	-34.419417243	150.814277788	1	<1	Female	Good
49	29/10/19	-34.419417243	150.814277788	0.8	<1	Male	Good
50	2/11/19	-34.715252112	150.539797492	6.7	3 to 4	Male	Good
51	2/11/19	-34.730950245	150.533271811	5.4	2 to 3	Female	Good
52	2/11/19	-34.728835783	150.534361075	6.2	3 to 4	Male	Excellent
53	9/11/19	-34.752005469	150.556783833	3.4	1 to 2	Female	Good
54	9/11/19	-34.750388224	150.558852082	1.2	<1	Male	Good
55	20/11/19	-34.678404434	150.591043014	6.5	4 to 5	Male	Excellent
56	22/11/19	-34.676148024	150.599587367	1.1	<1	Female	Good
57	22/11/19	-34.675798320	150.598768119	6.2	4 to 5	Male	Fair
58	22/11/19	-34.672290065	150.600986345	4.5	1 to 2	Female	Good
59	22/11/19	-34.668572384	150.603101296	2	<1	Male	Excellent
60	22/11/19	-34.681196007	150.611948086	4	1 to 2	Female	Good
61	22/11/19	-34.682811770	150.612512819	0.8	<1	Male	Good

Individual	Date	Latitude	Longitude	Weight (kg)	Age range (years)	Sex	Condition
62	13/12/19	-34.763070016	150.498376300	1.2	<1	Male	Good
63	13/12/19	-34.763192880	150.502163099	1.3	<1	Male	Good
64	13/12/19	-34.736014354	150.512340234	4.7	1 to 2	Female	Good
65	13/12/19	-34.726470776	150.544913507	1.2	<1	Female	Good
66	8/7/19	-34.883224772	150.623105173	7.8	>5	Male	Excellent
67	8/7/19	-34.883803827	150.621830394	6.3	3 to 4	Male	Good
68	8/7/19	-34.876168479	150.610356705	6.5	3 to 4	Male	Excellent
69	8/7/19	-34.872396263	150.625790686	7	4 to 5	Male	Good
70	27/12/19	-34.727150425	150.544194583	1.5	<1	Male	Good
71	27/12/19	-34.726366450	150.535733790	6.5	3 to 4	Male	Good
72	27/12/19	-34.727162263	150.543440820	4.1	1 to 2	Female	Good
73	27/12/19	-34.720944757	150.534213360	6.5	3 to 4	Male	Excellent
74	21/1/20	-34.871288072	150.615234279	4.6	<1	Female	Good
75	21/1/20	-34.877910890	150.618291845	5	2 to 3	Male	Good
76	21/1/20	-34.885543780	150.625521791	3.9	<1	Male	Good
77	21/1/20	-34.884875482	150.626383414	3.5	<1	Male	Good
78	25/1/20	-34.736302652	150.512331589	4.4	<1	Male	Good
79	25/1/20	-34.724847996	150.527535271	4.6	<1	Male	Good
80	25/1/20	-34.731094556	150.529718681	6.5	2 to 3	Male	Excellent
81	25/1/20	-34.716814158	150.538135391	3	<1	Male	Good
82	28/1/20	-34.890804164	150.505197237	4.8	<1	Male	Good
83	28/1/20	-34.893647339	150.506249091	5	<1	Male	Good
84	30/1/20	-34.881947918	150.486638402	4.8	1 to 2	Male	Good
85	4/2/20	-34.717737648	150.512604190	4.1	1 to 2	Female	Good
86	4/2/20	-34.735404899	150.543589897	4.2	1 to 2	Female	Fair
87	4/2/20	-34.739489553	150.544528245	4.5	2 to 3	Female	Good

Individual	Date	Latitude	Longitude	Weight (kg)	Age range (years)	Sex	Condition
88	4/2/20	-34.725439711	150.552357881	3.5	<1	Male	Excellent
89	13/2/20	-34.736493050	150.511943674	6	3 to 4	Male	Excellent
90	20/2/20	-34.920093973	150.386539111	3	<1	Female	Good
91	24/2/20	-34.716064113	150.498679995	3.5	<1	Male	Excellent
92	24/2/20	-34.718958770	150.498723788	4	<1	Female	Good
93	25/2/20	-34.737710645	150.511972676	4	<1	Female	Good
94	28/2/20	-34.751638949	150.556488809	5.9	3 to 4	Male	Fair