Landscape Genetics for Conservation Management: Brushtail Possums (*Trichosurus vulpecula*) in New Zealand

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Conservation Biology

Massey University

New Zealand

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2019

Abstract

The negative impact of brushtail possums (Trichosurus vulpecula) on New Zealand ecosystems became apparent soon after their introduction from Australia in 1858. Possums not only denude native vegetation but prey on native birds and invertebrates. They also carry bovine tuberculosis (TB) impacting the dairy industry and consequently the New Zealand economy. New Zealand possum populations have spread from several introduction sites and densities have increased. The resulting complex patterns of gene flow influences regional diversity, and potentially the effectiveness of control measures. Currently, ~100 million dollars are spent on 1080 management per year, mostly in response to Tb risk, but there is little information about the migration rates associated with resulting population density fluctuations. To determine whether the potential for intermixing between populations since their introductions could have caused a homogenizing effect on the genetic diversity across New Zealand, I began a detailed population genetic analysis by genotyping possums from 19 locations using nuclear microsatellites and mitochondrial DNA haplotyping from across the country to estimate population structure. Initial introductions of possums from multiple locations resulted in genetic and fur colour diversity but, in comparison to natural Australian populations, it appears that only a subset of genetic variants was brought to New Zealand from Australia. Mitochondrial sequence variation analyses showed overall high haplotype diversity with substantial differences among samples in haplotype frequencies, but with relatively low nucleotide diversity.

Similarly, analysis of nuclear markers (microsatellite genotypes with Naïve Bayesian clustering) reveals that while there has been admixture between populations in various locations, indicated by shared genotypes, there are genetically distinct regional populations. Concordance of genetic and geographically distant sampling shows a well-developed population structure of possums across New Zealand. These results are also supported by pairwise F_{st} comparisons between all pairs of populations; although nearly all populations showed significant differences, there was no signature of isolation by distance as expected from their history of introductions.

This study provides a foundation for further research into spatial structure of brushtail possums which will enable the effective targeting of management and is essential for modelling population recovery, disease spread, and potentially the emergence of toxin resistance. Predator-free 2050 is an ambitious objective considering current circumstances. In order to achieve its goals, even for the targeted species, we need to efficiently manage our resources and improve the accuracy of control measures to maintain long-term effects.

Acknowledgements

First and foremost, Steve and Mary, my amazing supervisors. Thank you for making my introduction to the world of research an absolute pleasure. For being so supportive, patient and enthusiastic through the course of this project.

Thanks to everyone in the Phoenix group for your help and inputs.

Emily, for being my guiding light- right from lab work to reading my chapters- your kindness and friendship saw me through some tough moments.

David, for knowing so much about software when I didn't.

The boys- Mat and Jules- for constantly entertaining me with your unusual sense of humour and for the birthday whiskey.

Lucy Cramp, my number one twin, for the pep talks, endless tea breaks, road trips and job opportunities. No one catered to my emotional needs as much as you did.

Kat, inhabitants of office 1.35 and my other postgrad friends from the department. I'll always be grateful for the positivity and interesting interactions we've shared, but most importantly for sharing your experiences and assuring me that I wasn't alone in my struggles.

I'm especially grateful to the researchers, sample collectors, academics and technicians who've supported this project by providing me with information, suggestions, encouragement and possum ears. It really puts into perspective how we're all a part of something bigger than ourselves.

Finally, I'm forever grateful to my wonderful family and friends back at home. Your love and support made it across the 11,603 kms. Mom and dad, thank you for the Sunday skype sessions that kept me from being homesick. All the success and happiness I experience is because of your love and stellar parenting skills.

Contents

LIST OF FIGURES

.

LIST OF TABLES

Chapter 1. General Introduction

Conservation Biology and Invasive Species

The introduction of invasive mammalian predators has resulted in the extinction of ~58% of global bird, mammal and reptile species worldwide (Brockerhoff et al., 2010; Doherty, Glen, Nimmo, Ritchie, & Dickman, 2016). As one of the foremost causes of loss in biodiversity, preventing the introduction of invasive species is imperative for conservation (Allendorf & Lundquist, 2003). Species that have adapted to life with few predators, such as flightless birds on islands, are the most sensitive to the effects of invasive predators. To prevent irreparable loss in species and phylogenetic diversity, the eradication of invasive predators should be high on the priority list for global conservation (Doherty et al., 2016).

An invasive species can be classified as one that has interspecific superiority (either in resource use and/or predation) that gives it an advantage in the novel ecosystem it has been introduced to (Valéry, Fritz, Lefeuvre, & Simberloff, 2008). These species expand their natural ranges via natural factors like wind and ocean currents and range expansion, usually after humans have aided long distance dispersal. (Olden, Lockwood, & Parr, 2011). The impact an invasive species has on the ecology of the new environment could be influenced by its ability to cope with the environmental conditions (Hooper et al., 2005); greater evolutionary potential is sometimes a consequence of higher levels of genetic diversity which is driven by the magnitude of introductions, including the methods of introduction (natural range expansion, artificial dispersal) (Dlugosch & Parker, 2008). Following establishment, the spread of invasive species happens naturally, especially if there is little competition for resources and the absence of their own natural enemies to limit population growth (Allendorf & Lundquist, 2003).

Survival in a new environment depends on many factors and attempting to limit the range or abundance of a pest species is a conservation challenge. The natural dispersal of individuals and barriers to spread of wild populations can be inferred from genetic diversity and population structure. If one documents patterns of genetic diversity on a spatial and temporal scale this can help design more effective control and management plans (Sakai et al., 2001). Fundamentally, landscape genetics deals with identifying the spatial patterns of population structure and movement, and the landscape features that could influence it (Richardson, Brady, Wang, & Spear, 2016).

Brushtail possums (Trichosurus vulpecula) are mammalian marsupials native to Australia (Pracy, 1974). Multiple anthropogenic introductions of possums to New Zealand since the

mid-1800s have resulted in their establishment and expansion, as they dispersed both naturally and via human-aided artificial dispersal (Pracy, 1974); resulting in approximately 70 million possums that inhabit the country today. Normally, when a species is introduced to a new environment, it represents only a subset of the total genetic variation present in the native populations resembling a bottleneck event on introduction (Sakai et al., 2001). Because each population established in New Zealand grew rapidly, the bottleneck was limited to a single generation limiting the expected loss of genetic diversity (Frankham, 2010). After the first successful release of possums into New Zealand, additions to establishing possum populations occurred in small numbers, but multiple times, which (if successful) is expected to elevate population genetic diversity (Clout, 2000; PE Cowan, 2005; Frankham et al., 2017). There is a relatively well-recorded introduction history of possums from Australia to New Zealand (Pracy, 1974). One study that used allozyme loci for the purpose of taxonomic clarification between four subspecies source populations of possums in Australia found that genetic divergence between the populations was low compared to subspecies of other mammalian counterparts (Triggs, 1989). Genetic studies of possums in New Zealand have analysed small-scale population densities in different environments, population structure and connectivity, and using this information to improve eradication strategies (Adams, 2013; Ramsey et al., 2002; Rouco, Norbury, Smith, Byrom, & Petch, 2013). Another study incorporated GIS data with microsatellite DNA data of possums in the Hawke's Bay region of New Zealand to identify a contact zone between two colour morphs of possums, potentially leading to novel genetic forms absent in Australia, and affecting their resistance to 1080 (Sarre et al., 2014). One large-scale study conducted genetic analyses with microsatellite markers in brushtail possums belonging to New Zealand and Australia, determining the genetic variation and phylogenetic relationship between the two (Taylor et al., 2004).

As an invasive, generalist species in an insular environment with the absence of their predators and parasites, possums have high densities in New Zealand. The negative impacts that possums have on the New Zealand ecosystem are apparent in the loss of native biodiversity, overuse of resources and spread of disease, all invariably affecting the country's economy (Goldson et al., 2015; Livingstone, Hancox, Nugent, & de Lisle, 2015; O'Donnell, 1995). Therefore, knowledge of origin of the species, their establishment and spread over the course of a century with the help of genetics and population biology tools is valuable in developing effective management plans to control their populations and minimise further devastation (Sacks, Brazeal, & Lewis, 2016).

1.1 Possums in New Zealand - Background Information

1.1.1 History of Possum introduction and control

Brushtail possums (Trichosurus vulpecula) were introduced to New Zealand in the mid1800s to establish the fur industry. However, multiple introductions were required until possum populations were successfully established across the county (Pracy, 1974). Thirtyfive introductions by acclimatization societies and the New Zealand government were recorded up until 1895, followed by a dramatic increase of ~127 releases until 1926 (Figure 1.1) (Pracy, 1974) .Trapping and shooting possums for fur began in 1900 and continued for the next few decades (with ban on unlicensed shooting); simultaneously, research into the ecological impacts of possums showed they had negligible effects on their ecosystem (Kirk, 1920).By 1922, the liberations of possums from Australia and other regions within New Zealand ceased. In the 1940s the negative effects that possums were having on the native flora and fauna were becoming obvious (Payton, 2000). Backed up by scientific evidence in 1940, brushtail possums were considered a pest species which led the government to lift all restrictions to kill possums by 1947, including the use of poison (Morgan & Hickling, 2000).

From 1950-1960, a bounty system was ordered causing ~1 million deaths of possums every year. Possums were known to damage endemic mistletoes, reduce forest growth and were photographed eating birds eggs and chicks in nests (Sadleir, 2000). In 1987, the Department of Conservation begun active control of possums under the Wild Animal control Act 1977 (Clout, 2000). When possums were recognised as carrying and transmitted bovine TB then more money was spent reducing possum densities in regions with dairy and cattle farming (de Lisle, 1993).

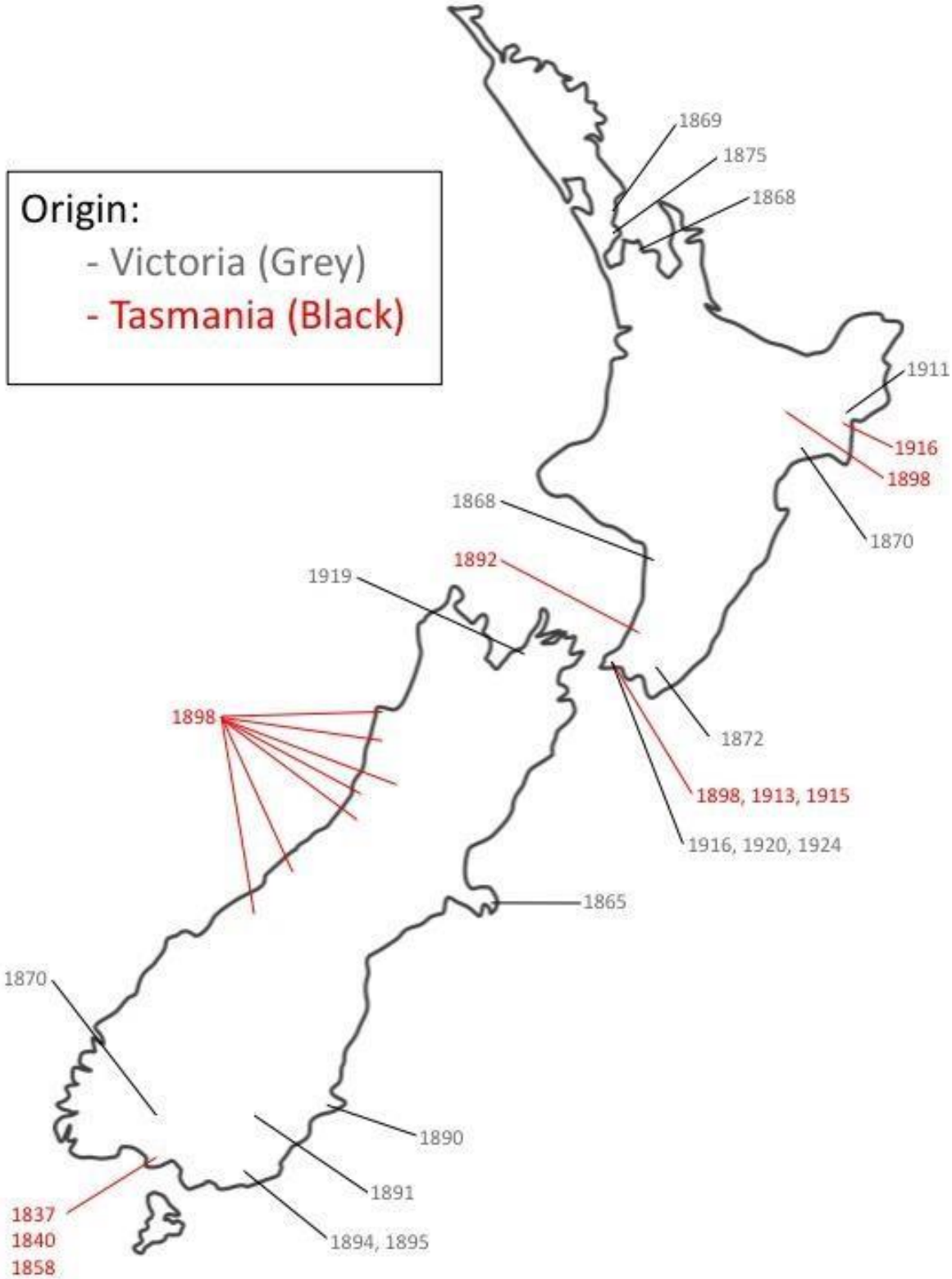

Figure 1.1: Introduction events of brushtail possums (*Trichosurus vulpecula*) to New Zealand based on records from Pracy (1974). Grey labels represent grey possums from mainland Australia (Victoria) and red labels represent predominantly black possums from Tasmania.

1.1.2 Origin

Brushtail possums (Trichosurus vulpecula) are naturally found in the mainland regions of Australia, Tasmania and the neighbouring islands (Pracy, 1974). Acclimatization societies across New Zealand believed that possums would contribute to the "artificial enrichment" of the country (McDowall, 1994). Possums were introduced from Australia by these acclimatization societies, the government and private owners (Clout, Ericksen, & Montague, 2000), thus there were simultaneously many populations being introduced, complicating their genetic make-up across within New Zealand.

In Australia, brushtail possums were not only geographically widespread, but their distinct coat colour and body size led them to be classified into a number of subspecies (Pracy, 1974). Previously classified into five subspecies based on their coat colour and geographic range: Northern (Trichosurus vulpecula arnhemensis), copper-coloured (T. v. johnsonnii), Tasmanian (T. v. fulginosus), South-western (T. v. hypoleucus) and South-eastern (T. v. vulpecula). Now they are all classified under the umbrella, Trichosurus vulpecula. Fur colours of possums from Tasmania were predominantly black and black-red while those from the Victoria were mostly grey, however, no evidence for considering possums from the mainland Australian and Tasmania as distinct subspecies populations was found using nuclear genetic markers (allozyme loci) (Triggs, 1989).

The first New Zealand introduction happened in Riverton, Southland in 1837, but the population did not establish. In 1858, however, another attempt in the same region established the first successful population- mainly to establish a fur industry in New Zealand (Figure 1.1). Majority of the introductions and translocations happened between 1858 to 1898 with restricted introductions between 1915 to 1925. Pracy (1974) gave a decadewise, detailed account of possum liberations in New Zealand and mentioned that the years of 1890-1900, acclimatization societies maximized their artificial dispersion activity.

Possums introduced to New Zealand from Australia were chosen based on preferred fur quality, i.e., possums with predominantly black or reddish black coats from Tasmania, those that lived in colder regions that had a denser, better quality coats were chosen. Only these colour morphs were chosen till 1915. Between 1915 to 1940, there was an increased demand for grey possums by private individuals and some organizations to improve possum breeds, improving fur colour choices, which invariably led to the depreciating value of the black, brown and reddish-brown colour morphs (Pracy, 1974).

In his expert opinion, biologist Harold Kirk wrote that possums did negligible effects on native forests and any damage was worth the cost as New Zealand's revenue had increased from the fur trade (Kirk, 1920). Following this, the greatest number of illegal translocations of possums

took place between 1922 and 1940. By 1940, the negative impact of possums on the environment became evident and support for further acclimatization had stopped (Pracy, 1974). By 1950s, heavy penalties for possum liberations were enforced and research for improved technology in control of possums were encouraged.

The establishment and extent of colonization of possums since their introduction was an unexpected success which then led to 35 to 70 million possums in New Zealand there are today.

1.1.3 Possum Biology

Brushtail possums in New Zealand are primarily of two fur colours- Black and Grey (Triggs & Green, 1989). Possums from Central Australia tend to be bigger and heavier than Tasmanian possums, with slight differences between males and females in size, weight and intensity of fur colour (PE Cowan, 2005). Adults have heights that range between 60 to 95 cm and weights between 1.5-7 kg (PE Cowan, 2005).

Possums are found in most major habitat types ranging from farmlands to the forests of Fiordland with high mountainous regions bring the only regions they do not occupy (Ruapehu, Western Fiordland) owing to the unsuitable climatic conditions at these heights (PE Cowan, 2005). They were also introduced to some off-shore islands, however they were eradicated from Kapiti Island in 1992 (Brown & Sherley, 2002). In tune with their introduction histories, possums from mainland Australia occupy areas that have denser vegetation (hardwood forests, exotic grasslands) while possums from Tasmania occupy open, drier regions (PE Cowan, 2005). During the day possums occupy dens in hollows of trees or areas above ground level that are normally well hidden (P Cowan & Clout, 2000).

Brushtail possums have their biggest impact on New Zealand biodiversity as folivores (Nugent, Sweetapple, Coleman, & Suisted, 2000). Over-browsing may sometimes cause a long-term change in the vegetation cover, but possums being generalist species, adjust their diets accordingly (Nugent et al., 2000). One study showed that diets of possums vary depending on the availability of the sources and therefore, as abundance of one source decreases, they adapt quickly by feeding on another (Owen & Norton, 1995). Physiology of possums requires them to consume high-energy foods which are not always obtained from a leaf diet. Therefore, they have adapted as generalists, which in New Zealand ecosystems, allows them to become opportunistic feeders and consume a variety of fruits, invertebrates and insect larvae. In extreme conditions of starvation, they resort to feeding on deer or possum carcasses (also increasing risk of contracting Tb) (Nugent et al., 2000).

Average life expectancy of possums ranges from six to ten years (Fletcher & Selwood, 2000). Possums are not monogamous, and females mature at 1-2 years of age and generally breed once a year (Kerle, 1984). Good body condition and environmental factors like availability of resources cause possums to breed twice a year (Fletcher & Selwood, 2000). Brushtail possums are marsupials with a short gestation period of about 18 days. The young are then born with developed internal organs, but underdeveloped hind limbs (PE Cowan, 2005). They further develop in the pouch for 120 days and become increasingly independent for 150 more days (Fletcher & Selwood, 2000). There is a heavy sex-bias in that more males are born than females (PE Cowan, 2005).

Just as in reproduction, there is a male sex-bias in their dispersal tendencies. Long-distance dispersal (key to establishing populations and disease transmission) involves as much as 10km per week (PE Cowan, Brockie, Smith, & Hearfield, 1997), with maximum movement and activity happening after dark as they are a nocturnal species (P Cowan & Clout, 2000). Female possums tend to take over their mothers' dens or overlap with their home ranges. Juvenile males, however, gradually move away from their natal range, making a number of moves before settling down in one location (P Cowan & Clout, 2000). Additionally, dispersal from natal ranges are independent of population density, which also throws light on the higher dispersal tendencies of juveniles compared to adult possums(PE Cowan, 2005).

In terms of spread of bovine TB, dispersal of females could also play an important role as they make longer moves, if not as many, as male possums (P Cowan & Clout, 2000). Studies show that there is a greater likelihood of possums from adjacent home ranges taking over dens in possum-controlled areas than possums located at a greater distance (Brockie et al 1991; Efford et al 2000).

1.1.4 Possums, the problem

In Australia, brushtail possums occupy mostly eastern and northern parts along with Tasmania and other neighbouring offshore islands (Figure 1.2). They were mostly widespread in arid regions but faced a decline in numbers over the last century due to European settlements and predators (Kerle & How, 2008).

Possum population densities depend on foraging opportunities (Efford, Warburton, & Spencer, 2000). New Zealand offers a broad range of habitat types including hardwood forests, exotic grasslands, farmlands and montane scrublands (PE Cowan, 2005). Primarily folivores, a study by Nugent et al (2000) summarised the contents of possum diets where it showed that in hardwood forests, foliage makes up the majority of their diets. Possums are known to consume roughly 21,000 tonnes of foliage per day, sometimes leading to complete defoliation of canopy species in a very short period of time (Nugent et al., 2000).

Problematically, in native forests, the endangered tree species- northern rata (Metrosideros robusta) and southern rata (Metrodieros umbellata)- form a majority of possum diets, which are habitats for native bird (Nugent et al., 2000). They are known to feed on 70 species of native trees and 30 species of exotic forest cover (PE Cowan, 2005). They are known to consume native birds like the kiwi (genus Apteryx), kokako (Callaeas), and kereru (Hemiphaga novaeseelandiae) and their eggs which is a major threat to their declining populations (Nugent et al., 2000).

Their numbers are kept in check by predators like dingoes (Canis lupus dingo), foxes (Vulpes vulpes), pythons etc (PE Cowan, 1990). Additionally, harmless prey, such as native invertebrates, native birds and their eggs and snails, provide them with an abundant source of food, making it easier for them to thrive (Nugent et al., 2000).

Brushtail possums in Australia were noted to have many parasites (PE Cowan, 2005), of which only 14 (2 not Australian) managed to remain in the New Zealand populations. This reduces the burden on the population survival effort, enabling their populations to disperse, develop and reproduce (Clout, 2000).

Finally, the climatic conditions of New Zealand compared to the more arid regions of Australia, allow for vegetation growth that is both abundant and more than adequately meets their nutritional requirement, thus supporting the browsing activities of many more individuals relative to Australian vegetation (Pracy, 1974). It is clear, had there been more stringent precautionary measures taken at the time of their introduction, the country wouldn't be dominated by the ~70 million possums there are today (Clout, 2000).

Figure 1.2: Distribution of brushtail possums (*Trichosurus vulpecula*) across Australia according to IUCN (2016).

1.1.5 Bovine tuberculosis (Tb)

The biggest negative impact that possums are associated with is the spread of Bovine Tuberculosis (Tb) in New Zealand. Attention was brought to them in the 1960s when it was discovered that possums are a reservoir for Mycobacterium bovis that cause Tb in cattle and deer (Livingstone et al., 2015).

Bovine Tb was first detected in cattle in the 19th century in parts of Europe- which spread to other parts of the world, including New Zealand, due to illegal, unrestricted trade of cattle (Livingstone et al., 2015). Once detected in New Zealand populations in the late 1800s, all cattle were subject to inspection and slaughtered if infected with the bacteria (Livingstone et al., 2015). By 1967, possums were discovered to be vectors (direct transmitters) of Tb to cows, pigs and deer (Livingstone et al., 2015). However, in Australia, possums are not a vector of the disease which may be because Tb has been eradicated in Australian cattle and thus possums haven't contracted the disease from them and that possums have had minimal interactions with cattle in Australia in the past (Landcare Research, 2000). When possums are infected with Tb, they develop lesions (affect lymph glands and produce lime-green pus), which invariably infect livestock either by direct transmission or contact with infected surface (Livingstone et al., 2015). About 60% of possums in New Zealand are infected and are

expected to survive for up to 6 months after showing clinical symptoms of infection (PE Cowan, 2005). The spread of Tb was mostly in contiguous forests and farmlands; with an increasing spread noticed in the paddocks between two forested areas (Livingstone et al., 2015). Thus, with the multiple introductions and movement rates of the possums, bovine Tb spread rapidly increasing the rates of infection in cattle across New Zealand.

1.1.6 Sodium fluoroacetate (1080)

Sodium fluoroacetate is a chemical compound that is used in New Zealand to kill possums and other mammalian pest species (Triggs & Green, 1989). Fluoroacetate is naturally produced in some poisonous plants in Australia and other parts of the world (Eason, 2002). First developed in the United States in 1940s as a rodenticide prior to the Second World War, it was used to target mammalian pests such as gophers, prairie dogs etc, bringing it to use in New Zealand since the 1950s (Eason, Miller, Ogilvie, & Fairweather, 2011). Its use to target vertebrate pests in a country like New Zealand is well justified because the only other native mammalian species are the Greater Short-tailed bat (Mystacina robusta) and Lesser Short-Tailed bats (Mystacina tuberculate) with no other native fauna being affected; which is unlike countries where non-target mammalian species might consume this fatal bait (Eason et al., 2011). Brushtail possums in Tasmania are believed to show more resistance to the toxin 1080 at lowered temperatures (McIlroy, 1983) as it is a component naturally present in plants in Australia.

The highly soluble and biodegradable nature of 1080 doesn't allow it to reach measurable quantities in water bodies. This, along with substantial evidence that indicates minimal risk to population-level survival of native birds makes New Zealand the largest user of 1080 in the world for mammalian pest control (Eason et al., 2011; Veltman & Westbrooke, 2011). On the other hand, livestock are quite susceptible to the baits and therefore, when applied around farms, death of cattle have been noticed when they were brought back to those areas less than 5-10 days post-treatment (Eason et al., 2011), which is an issue that can be avoided considering the advantages 1080 has on conservation of native biodiversity.

According to the DoC map indicating 1080 target areas (Figure 1.3), we see that most pesticide control has been targeted in the Marlborough and West coast regions and otherwise around National parks and protected land. Aerial drops and bait stations have been the most common methods of 1080 treatment thus far, but there have been no studies analysing the post-treatment response in these locations. There has been no strong genetic basis for targeting these particular locations- only the presence of native species and dairy farms- which invariably affects the economy.

New Zealand Institute of Economic Research reported in 2017 that dairy ($13.6 billion/year) followed by meat ($6 billion/year) are the country's biggest export products (as of 2016) (Corong, 2014) with the total value of possum industry being ~$127 million/year (Corong, 2014). Clearly, the value they add to the country's economy is negligible compared to the damage they are causing to it as well as to the native flora and fauna (Section 1.1.4).

The crux of 1080 treatment to control possums is to prevent the hit that the economy is taking, considering dairy and beef are the biggest export industries in New Zealand, but from the conservation point of view, it is important to rid New Zealand of these mammalian pests before native trees and bird populations go extinct.

1.1.7 Current methods of possum control and Tb eradication

Presently, the possum fur industry contributes approximately $130 million/year to the New Zealand economy with 2 million possums being killed per year (Corong, 2014). This number is small in comparison to the current possum numbers inhabiting the country and negatively impacting native biodiversity.

The Department of Conservation (DoC) and the Animal Health Board (AHB) are the two major organizations managing the eradication of possums followed by Regional Councils and private owners (NPCA, 2015). DoC predicts an increase to New Zealand biodiversity and economy (monetary savings) with increasing possum takes (Corong, 2014). For this purpose, most common methods to kill possums include baiting with 1080- aerial drops/ground bait stations, kill-traps and shooting.

Landcare Research produced reports indicating potential immunocontraception as biocontrol of possum populations. This involves effectively obstructing the production of a protein important to forming the outer layer of the ovum in females. This method has been successful in mammals like pigs and horses, but more research is required in the case of possums. Moreover, using a genetically modified viral strain may not be well-received by the public to go ahead with this method (Landcare Research, 2004).

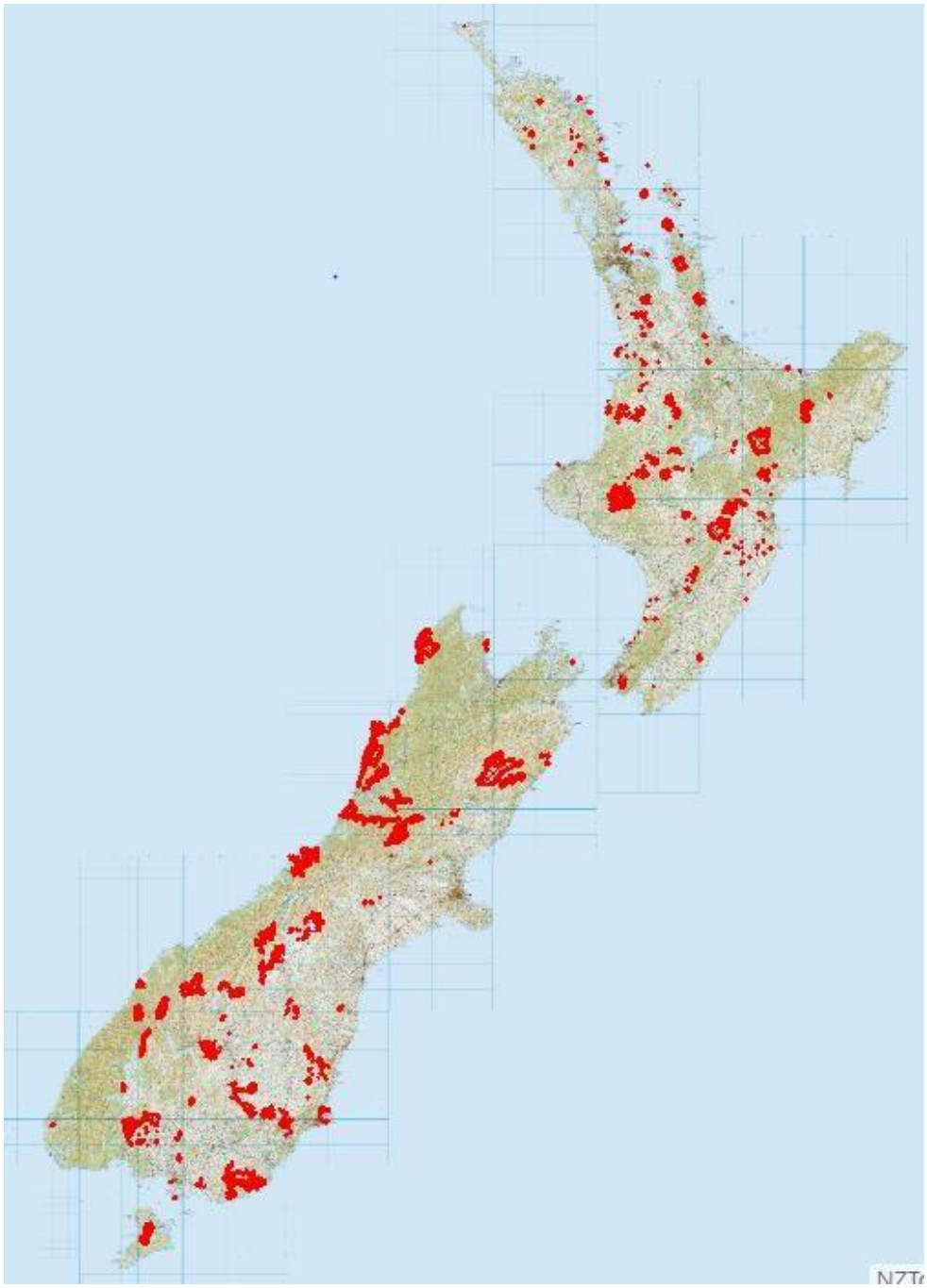

Figure 1.3: Areas across New Zealand subjected to 1080 bait control for possum management until 2017 (shown in red).

1.2 Thesis Structure

Chapter2: Population structure - Mitochondrial sequence variation - I document the haplotypic variation that exists among wild possum populations in Australia and New Zealand. DNA sequence of control region of the mitochondrial genome (single locus) for 209 individuals from 14 different locations, identified admixture patterns. Additionally, I created a haplotype network to visualise associations between morphological diversity (fur colour) and mtDNA haplotypes and to corelate this information with the introduction histories of possums to New Zealand.

Chapter 3 Population structure - Nuclear markers - I identify population structure and distribution of genotypic variation among possum populations using 275 individuals from 19 locations. For this, I used seven polymorphic nuclear microsatellite markers developed in previous studies and used a naive Bayesian modelling approach to cluster populations into genetic groups. I also examine genetic data for evidence of Hardy-Weinberg equilibrium, Isolation by distance and genetic differentiation. These analyses were conducted on my dataset and on raw data from the Taylor (2004) study. Providing information on population structure of New Zealand brushtail possums and comparing temporal samples.

Chapter 4 General Discussion -This thesis provides foundational information for population structure and the levels of admixture among New Zealand brushtail possums. Results suggest some genetic admixture between populations while retaining a well-developed population structure. Effective control of wild populations can benefit from knowledge of the geographic limits of populations and routes of dispersal that make connections between populations.

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Chapter 2. Mitochondrial Haplotype Diversity

2.1 Introduction:

The mitochondria has a circular genome that is inherited from the matriline and therefore, mutations are passed down from one generation to the next without any recombination (Harrison, 1989). DNA sequence variation of the mitochondrial genome has been used extensively in evolutionary studies of natural populations of plants and animals (MORITZ, 1994). The relatively rapid mutation rate, universal primers and high copy number, all facilitate studies of population structure over spatially and temporally small scales (Avise 1994). The non-coding control region of mtDNA is often polymorphic within species and aids such analysis by allowing the identification of intraspecific variation while making it sensitive to the effect of landscape or other environmental barriers (Chapman, 2001). Additionally, mitochondrial DNA is inherited as a haploid organelle and therefore, when used as genetic markers, represent only half the effective population (N_e) as nuclear markers (Sunnucks, 2000). The mitochondrial genome of the brushtail possum is ~17 kbp. For this study, I will amplify the non-coding control region (also known as D-loop) which is the most rapidly evolving segment of the mammalian mitochondrial genome (Janke, Feldmaier-Fuchs, Thomas, Von Haeseler, & Pääbo, 1994).

mtDNA markers have been used to study the population structure of mountain brushtail possums (Trichosurus cunninghami) in Australia (Blyton et al. 2012), but there is a paucity of information regarding haplotypic variation within and between populations of brushtail possums (Trichosurus vulpecula) in both their native range and new home in New Zealand. There has been documentation of genetic diversity of brushtail possums in New Zealand using nuclear markers (Adams, 2013; Dueñas, Cruickshank, & Ross, 2015; Sarre et al., 2014) (Taylor et al., 2004). Given the numerous documented translocations of possums to New Zealand for the purpose of hunting and the fur trade, there is opportunity for using mtDNA variation to examine population structure and history (Pracy, 1974).

Here, I document mtDNA haplotype variation from brushtail possums collected from the wild, in Australia and New Zealand. This is the first study to analyse population structure based on haplotype diversity for brushtail possums on a large scale. The many source populations in Australia (including Tasmania) that were used to establish the current New Zealand population is expected to result in high haplotype diversity within New Zealand. However, because of the many introductions to New Zealand, their rapid expansion and dispersal abilities, I expected mixing of source populations would result in little genetic structure, with no population completely isolated from the others. However, the

consequences of recent management in New Zealand (killing) could be reflected in the loss of haplotype diversity from genetic bottlenecks for some populations.

2.2 MATERIALS AND METHODS:

2.2.1 Sample collection:

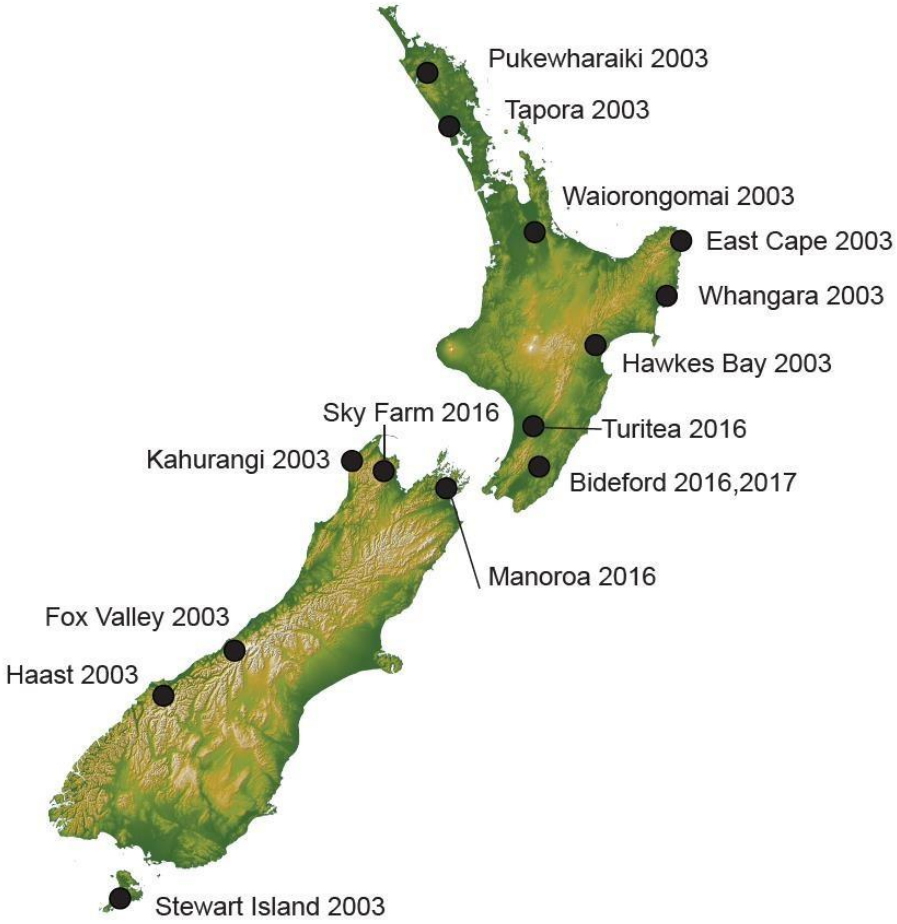

Figure 2.1: New Zealand sample sites for mtDNA analysis of brushtail possums *(Trichosurus vulpecula*)

Table 2.1: Locations and year of sampling for brushtail possums in New Zealand with individuals in each population sample and collectors in each location. Abbreviations of each location underlined as used further in the chapter.

Location	Number of samples	Year of sampling	Collector
Wairongomai	6	2003	Landcare Research
Manaroa	36	2016	Ralph Powelsland
Gisborne	10	2003	Landcare Research
Tapora	11	2003	Landcare Research
West Coast	18	2003	Landcare Research
Hawke's Bay	14	2003	Landcare Research
Greater Wellington Region (GWR)	28	2017	Murray Hudson
Kahurangi Point	8	2003	Landcare Research
Abel Tasman	12	2003	Landcare Research
Pukewharaiki	13	2003	Landcare Research
Stewart Island	20	2003	Landcare Research
Bideford	16	2016	Tim Brenstrum
Skyfarm	7	2016	Steve Trewick
Turitea	10	2016	Steve Trewick

Two hundred and twenty-five possum ear samples were collected from 14 locations across New Zealand between 2003 and 2016 (Figure 2.1; Table 2.1). There is a noticeable gap in sampling time (2003 to 2016. 2017 and 2018) as well as sampling location (Eastern South Island and Western North Island) which could alter the outcome of genetic analysis if the gaps were filled as management operations during this time could influence the distribution of possums. For simple interpretation of results, West Coast region consists of samples grouped together from Fox Valley and Haast Plains, both sampled in 2003.

Australian samples consisted of T. vulpecula samples from 6 locations of Western Australia.

Ear clippings were taken from dead animals and stored in 100% ethanol.

Sex and fur coat colour were recorded for 154 individuals.

Whole mtDNA genomes

In a separate project, Australian and New Zealand possum populations were sequenced using Next Generation Sequencing (NGS) and I used this data to develop primers that amplified the control region of the mitochondrial genome. Every individual read (forward and reverse) were paired using the "Set paired reads by name" tool then sequences were mapped to a reference; the annotated mitochondrial genome of Trichosurus vulpecula obtained from the NCBI database (AF357238). This allowed the assemble of the complete mitochondrial genome and identification of all genes (Figure 2.2).

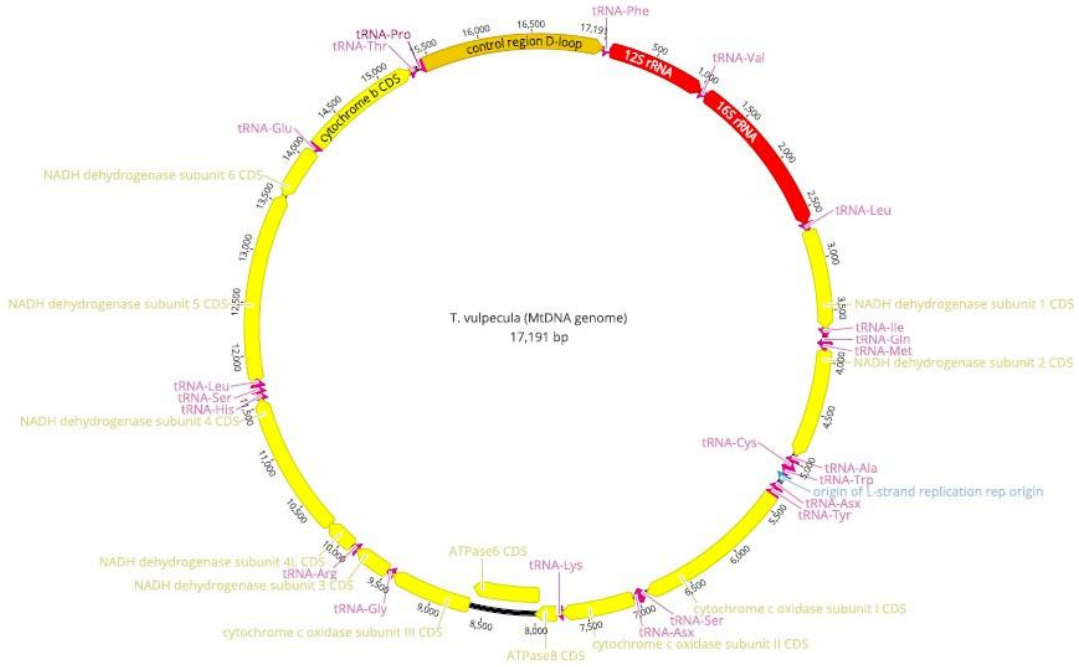

Figure 2.2: mtDNA whole genome of Brushtail possums (*Trichosurus vulpecula*). The control region (D-loop) is amplified for this study.

2.2.2 Laboratory methods:

DNA extraction was conducted using the GeneAid ™ Tissue DNA Isolation Kit following the manufacturer instructions with a final elution volume of 200µl. The quality of the DNA extraction was tested by loading 5µl of the DNA samples on a 1% electrophoresis agarose gel stained in SYBR safe. DNA concentration was evaluated using Invitrogen Qubit 4 Fluorometer (ThermoFisher Scientific).

Amplification of double-stranded DNA targeted the control region (~730 bp) of the mitochondria using primers designed in this study for this species (Table 2.2). Polymerase

Chain Reactions (PCR) were conducted in volumes of 20 µl which contained 2µl of 10x Dream Taq buffer, 0.4µl of deoxy-nucleotide phosphates (dNTPs), 0.5µl of 10µM forward primer (218 T.v.caninus), 0.5µl of 10µM reverse primer (1023 T.v.vulpecula) (Table 2.2 ; Figures 2.3 and 2.4), 0.1 µl DNA polymerase, 15.1µl water, 1µl of DNA extracted samples and an additional 0.4µl of Magnesium Chloride ($MgCl^{2+}$) (25mM). This mixture was then put into the Thermocycler under the following conditions: initial denaturation step at 95°C for 90 seconds, followed by 36 cycles of 94°C for 20 seconds, 51°C for 20 seconds, and 72°C for 1 minute; with a final 8-minute extension step of 72°C. PCR products were sequenced using BigDye® chemistry (Perkin Elmer) following the manufacturer's protocols on an ABI3730 DNA analyser (Macrogen).

D-loop primers for two sister-species of possums were developed- Tcan: Short-eared possums (Trichosurus canninus) and Tvul: Brushtail possums (Trichosurus vulpecula). Initially, I used the forward and reverse primers for Tvul, but the resulting sequences were of poor quality (Figure 2.5). Therefore, I amplified the D-loop sequence with the forward primer of Tcan (Figure 2.3) and reverse primer of Tvul (Figure 2.4). This resulted in cleaner reads, so I followed this protocol for all the samples.

Table 2.2: Primer sequences designed for this study to amplify control region mtDNA of two species of possums. Tcan- Short-eared possum (*T. canninus*); Tvul- Brushtail possum (*T. vulpecula*)

Primer name	Primer sequence
Tcan_218 (Forward)	AAGGCAACAACACCTCACCA
Tcan_1049 (Reverse)	AATACGACATCGGCGACCTC
Tvul_228 (Forward)	CACCTCACCATCAACACCCA
Tvul_1023 (Reverse)	TCCCGCCCAGTTGATAAACC

Figure 2.3: Position of the two forward primers for amplification of the possum D-loop region of the mitochondrial genome: Tcan_218F (*T. canninus*) and Tvul_228F (*T. vulpecula*).

Figure 2.4: Position of the two reverse primers designed for amplification of D-loop region of the possum mitochondrial genome. Tcan_1049R (*T.canninus*) and Tvul_1023R (*T. vulpecula*)

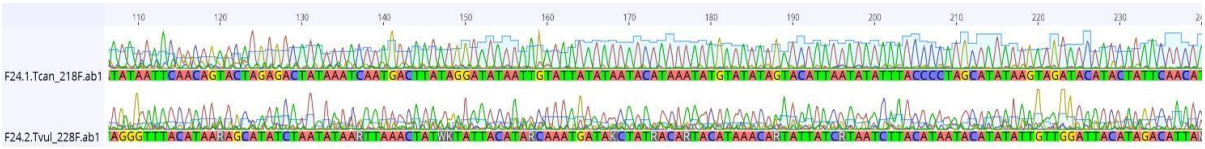

Figure 2.5: DNA sequence chromatograms produced using different primers: Tcan_218F (top read) and Tvul_228F (bottom read) from the same sample, amplified with the reverse primer (Tvul_1023R).

2.2.3 Population genetic analysis

DNA Sequence reads were checked and aligned by eye in Geneious v6.05 (Kearse et al. 2012) and trimmed to 732bp long. Two hundred and nine sequences were used to estimate population sample genetic diversity and related analyses. DNA polymorphism tests, haplotype diversity and population size change tests were conducted using the software DnaSP v 6.12.01. Data was exported from Geneious using nexus file format and grouped into 14 population samples. Information about the differences in the nucleotide sequences was computed for each population separately (Appendix 1) using DNA polymorphism tests in DnaSP. Overall haplotype (h) and nucleotide (π) diversity was calculated using a "MultiDoman Analysis" with the same. I sought evidence of population structure with haplotypic data by estimating genetic differentiation between the 14 population samples, with pairwise Φ_{st} and tested for significant deviations from zero using 100 permutations, with a 0.05 level of significance in Arlequin 3.5.2.2 (Table 2.4). A Φ_{st} value closer to 0 indicates higher levels of genetic similarity than those closer to 1.

To quantify the differences between all possible pairs of haplotypes, I conducted a Mismatch Distribution analysis (Figure 2.6) using DnaSP (Harpending, 1994). The raggedness of the resulting graphs can be used to infer growth of the population, with the position of the peaks indicating the time of population expansion, however this requires an assumption that the sample came from a single population without subdivision (Harpending, 1994) An overall analysis for the full dataset was conducted producing graphs that showed the distribution of observed pairwise nucleotide site differences (x-axis) against frequency (yaxis).

2.2.4 Haplotype network analysis :

In order to visualise the intraspecific relationship among the haplotypes, I created a haplotype median-joining network (Bandelt, Forster, & Röhl, 1999) using Popart (Leigh & Bryant, 2015). Such a representation is effective because the sequence length amplified is long enough to show differences among individuals and population studies frequently contain both ancestral and derived sequences that do not readily fit on a phylogenetic tree.

The "median-joining network" tool (Bandelt et al., 1999) in POPART contains additional median nodes between the haplotypes which are indicative of ancestral (missing) or unsampled sequences which make the network more straightforward thereby reducing the overall length of the network (Leigh & Bryant, 2015). Lines join each of the haplotypes (nodes) based on their similarity and each bar crossing these lines represents a base difference between the sequences.

209 sample sequences were categorised into their respective populations. To simply the interpretation of the network, GWR (2017) population was grouped with Bideford (2016) and other population samples were grouped into their respective regions which was colourcoded (Figure 2.7).

Using the mtDNA sequence, I created a dataset with only individuals where fur colour was known, including outgroup taxa. A median-joining network was inferred, dividing the individuals of the populations according to fur colour (Figure 2.8). The interpretation of this network is similar to the previous haplotype network, but instead of location each colour represents the proportion of one of the four possum fur colours (black, brown, grey and ginger) with the edges representing the differences between the sequences.

2.2.5 Phylogenetic Analysis:

A phylogenetic tree represents the evolutionary relationships among "haplotypes" was inferred. When haplotypes are representative of the whole genome, then the tree depicts relations among species and populations.

Haplotypes from Australian possums were added to the New Zealand haplotype alignment. The Australian samples included T. vulpecula samples from 6 locations within Western Australia and Tasmania, and as outgroups the two species T. caninus and T.cuninghani. As there were many individuals from each New Zealand location, I reduced each of these to one haplotype per population sample. This was to provide an overall representation of one population with respect to the others in the dataset. These Mitochondrial Control Region

sequences were then used to generate a Maximum Likelihood tree using Geneious 11.5 with 1000 bootstraps. The tree (Figure 2.9) contains 11 Australian mitochondrial control region sequences from different locations along with all the samples from my dataset.

2.3 Results

2.3.1 Population Genetic Analyses

The population variation dataset consisted of DNA sequences from 209 individuals which represented 30 distinct haplotypes (Table 2.3). Each sequence was 732 bp, with 55 polymorphic sites in the alignment, and overall nucleotide diversity (π) of 0.014 and haplotype diversity (h) of 0.82.

Of the 30 haplotypes, two were very common and seen in 57.9% of the sample (68 and 53; haplotypes 3 and 6). One or both of these common haplotypes were observed in 11 of the population samples (Table 2.3). However, many haplotypes were restricted to just a few individuals (21 of 30 haplotypes in < 4 individuals) or just one population sample ($n=14$). The population sample from West Coast ($n=18$) and Manaroa ($n=36$) had the highest π of 0.012 and 0.011 respectively. The Bideford population sample had the lowest π (0.0005). Population samples collected in geographical proximity did not necessarily show similar patterns of genetic diversity. For example, close to Bideford is GWR ($\pi= 0.002$) and Turitea ($\pi= 0.008$) with much higher levels of diversity by comparison. Haplotype diversity also varied (Table 2.3). To clarify this pattern of results, I ran a "Population size change" analysis in DnaSP. A mismatch distribution graph of frequency distribution showed an overall multimodal pattern of observed pairwise frequency distribution (Figure 2.6), not in correlation with the expected frequency distribution. Generally, multimodal mismatched distribution of a population indicates large populations with demographic equilibrium (Slatkin, 1987). Mismatch distribution analysis for all 14 population samples showed a multimodal pattern indicate either changing population sizes, more than a single incidence of introduction, or population subdivision. Smoothness of the observed distribution is unaffected by the population structure (Harpending, 1994).The analysis for individual population samples is shown in Appendix 3. The hypothesis of a single unstructured population of possums looks unlikely, so I estimated population differentiation. Most pairwise comparisons of populations revealed significant genetic differentiation as estimated by pairwise Φ_{st} (Table 2.4). However, there was no significant differentiation between 12% (11/91) of the population sample comparisons; GIS-HAW, TUR-MAN, SKYSTE, SKY-TUR and BID-GWR (Table 2.4). These genetically similar population pairs include comparisons of

North and South Island samples that had only the common haplotype (either haplotype 3 (SKY, TUR, STE) or haplotype 6 (GWR, BID).

Table 2.3: DNA polymorphism test showing the haplotypes that each location shares along with nucleotide (π) and haplotype (h) diversities. n= number of individuals in each location. The two most common haplotypes 3 (n=68) and 6 (n=53). Highest π= 0.012 (West Coast), highest h= 0.91 (Pukewharaiki).

		Haplotypes																													Diversity	
Location	**n**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	**n**	**h**
WES	18	5	5	1																6										1		0.012	0.77
WAI	6	3			2	1																										0.001	0.73
MAN	36	1		21			14																									0.011	0.52
GIS	10			5				3	1	1																						0.005	0.71
TAP	11										5	2	2	1	1																	0.003	0.78
HAW	14			7				5								1	1															0.005	0.66
GWR	28	1		1			26																									0.002	0.14
KAH	8			8																												0	0
ABE	12	9		1														1	1													0.002	0.45
PUK	13										2	1	1								4			1	1	1	1	1				0.006	0.91
STE	20			11																	7	1	1									0.005	0.6
BID	16						13																						3			0.0005	0.33
SKY	7			6																										1		0.001	0.28
TUR	10			7																									2		1	0.008	0.51
Total	209	19	5	68	2	1	53	8	1	1	7	3	3	1	1	1	1	1	1	6	11	1	1	1	1	1	1	1	5	1	2		

Table 2.4: Φ_{st} pairwise comparisons between possum population samples with significant values of differentiation in bold. Non-significant values in grey cells (12% of population sample comparisons).

	WES	MAN	KAH	ABE	STE	SKY	TUR	WAI	GIS	HAW	GWR	PUK	TAP
WES													
MAN	**0.14**												
KAH	**0.46**	**0.53**											
ABE	**0.23**	**0.32**	**0.86**										
STE	**0.24**	**0.23**	**0.67**	**0.186**									
SKY	**0.29**	**0.21**	**0.94**	**0.499**	0.05								
TUR	**0.13**	0.02	**0.65**	**0.286**	0.08	0.03							
WAI	**0.23**	**0.33**	**0.90**	**0.179**	**0.26**	**0.63**	**0.29**						
GIS	0.14	**0.22**	**0.60**	**0.265**	**0.12**	0.20	0.11	**0.28**					
HAW	**0.14**	**0.22**	**0.55**	**0.246**	**0.13**	0.18	0.12	**0.28**	0				
GWR	**0.62**	**0.45**	**0.89**	**0.867**	**0.79**	**0.87**	**0.71**	**0.87**	**0.82**	**0.79**			
PUK	**0.33**	**0.38**	**0.67**	**0.425**	**0.28**	**0.49**	**0.37**	**0.39**	**0.40**	**0.38**	**0.81**		
TAP	**0.52**	**0.51**	**0.88**	**0.766**	**0.58**	**0.79**	**0.58**	**0.78**	**0.67**	**0.62**	**0.86**	**0.27**	
BID	**0.67**	**0.50**	**0.98**	**0.944**	**0.84**	**0.96**	**0.78**	**0.96**	**0.88**	**0.85**	0.03	**0.85**	**0.93**

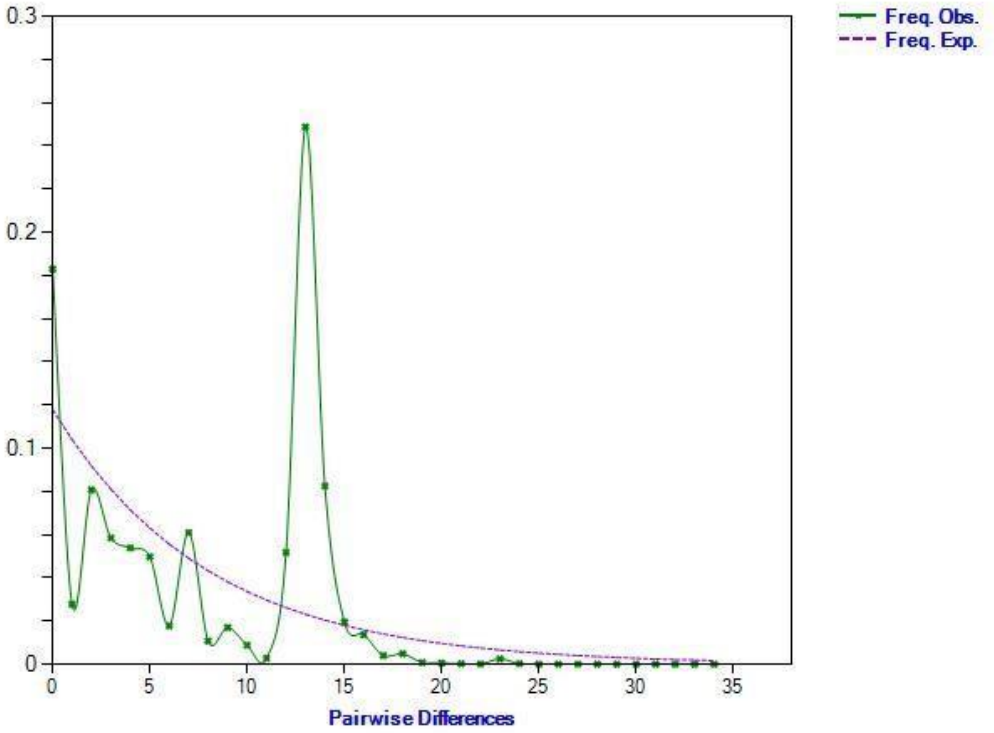

Figure 2.6: Mismatch distribution for all 14 possum population samples combined. The green line with dots represents the observed frequencies of pairwise differences and the purple line shows the expected values. More raggedness indicates higher rate of growth of the population and the position of the peaks indicate the time of population expansion (Harpending, 1994).

2.3.2 Haplotype Network Analysis

The distribution of mitochondrial variation reveals some geographic structure (Figure 2.7) when haplotype network is colour coded to reveal sampling location. The most common haplotype (3) is shared among all population samples except Bideford and populations in the north (Pukewharaiki, Tapora and Wairongomai); the next most common haplotype (6) is shared between Bideford, Manaroa and GWR. This representation is advantageous in that it not only reflects the results of the statistical analysis (Table 2.3), but also illustrates the genetic difference between the haplotypes. It is evident from Figure 2.7 that there is a quantitatively large nucleotide base pairs difference between haplotype 3 and 6. Haplotype 1 (19 individuals) is more closely related to haplotype 3 (2 nucleotide base difference) than to Haplotype 6. Few nucleotide differences between the other haplotypes are apparent. These

other haplotype nodes are smaller indicating fewer individuals, but even in this, there is no regional classification of haplotypes.

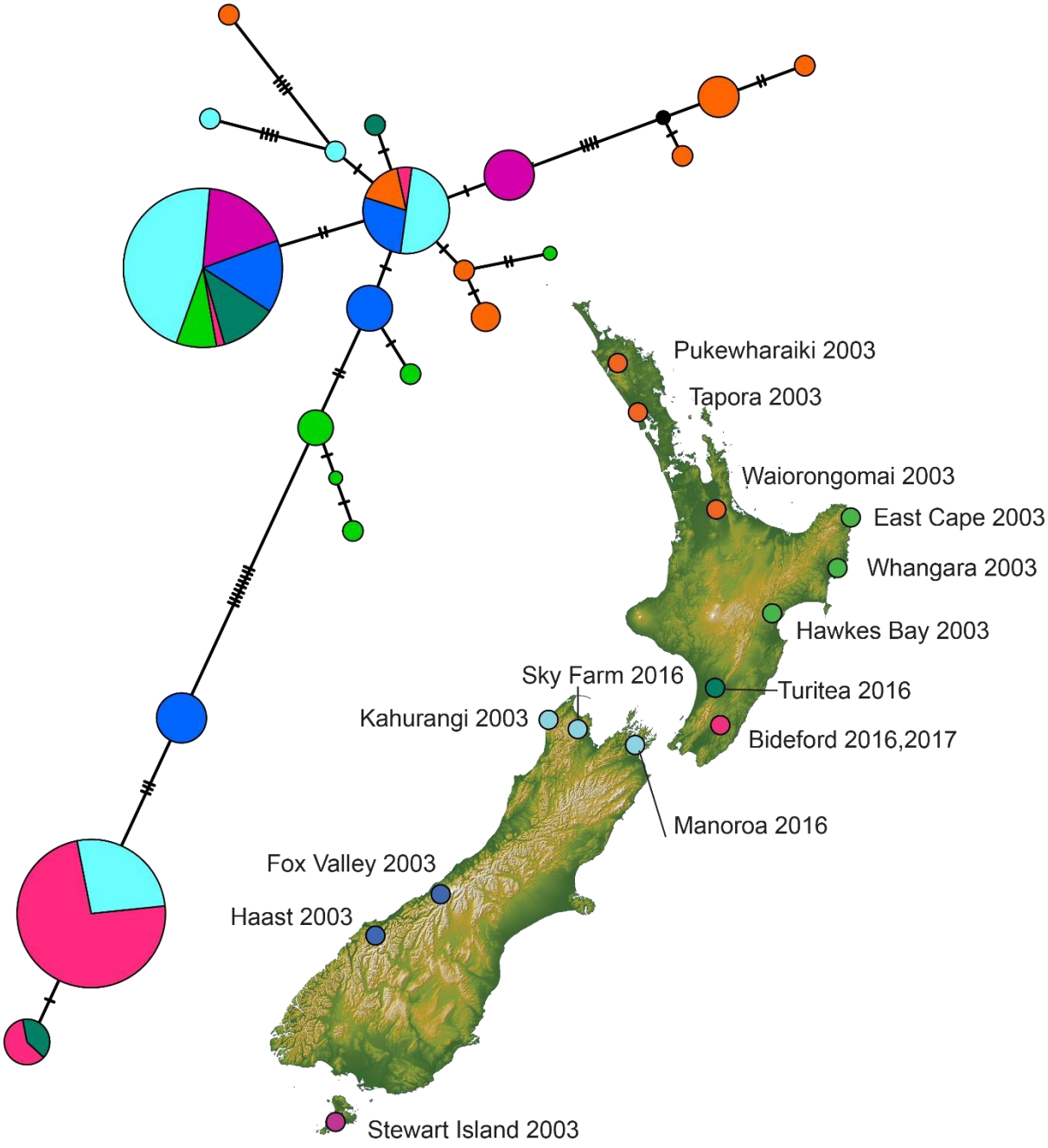

Figure 2.7: Haplotype network representing haplotype diversity of 14 locations (209 individuals) grouped according to region. Each circle represents a haplotype and each colour represents a region. The regional colour-codes include orange (Pukewharaiki, Tapora, Wairongomai), green (Gisborne, Hawke's bay) , teal (Turitea), pink (Bideford, GWR) , sky blue (Manaroa, Skyfarm, Abel Tasman, Kahurangi), deep blue (Fox Valley, Haast, Maruia, Lewis Pass) and purple (Stewart Island).

Haplotype diversity for fur colour

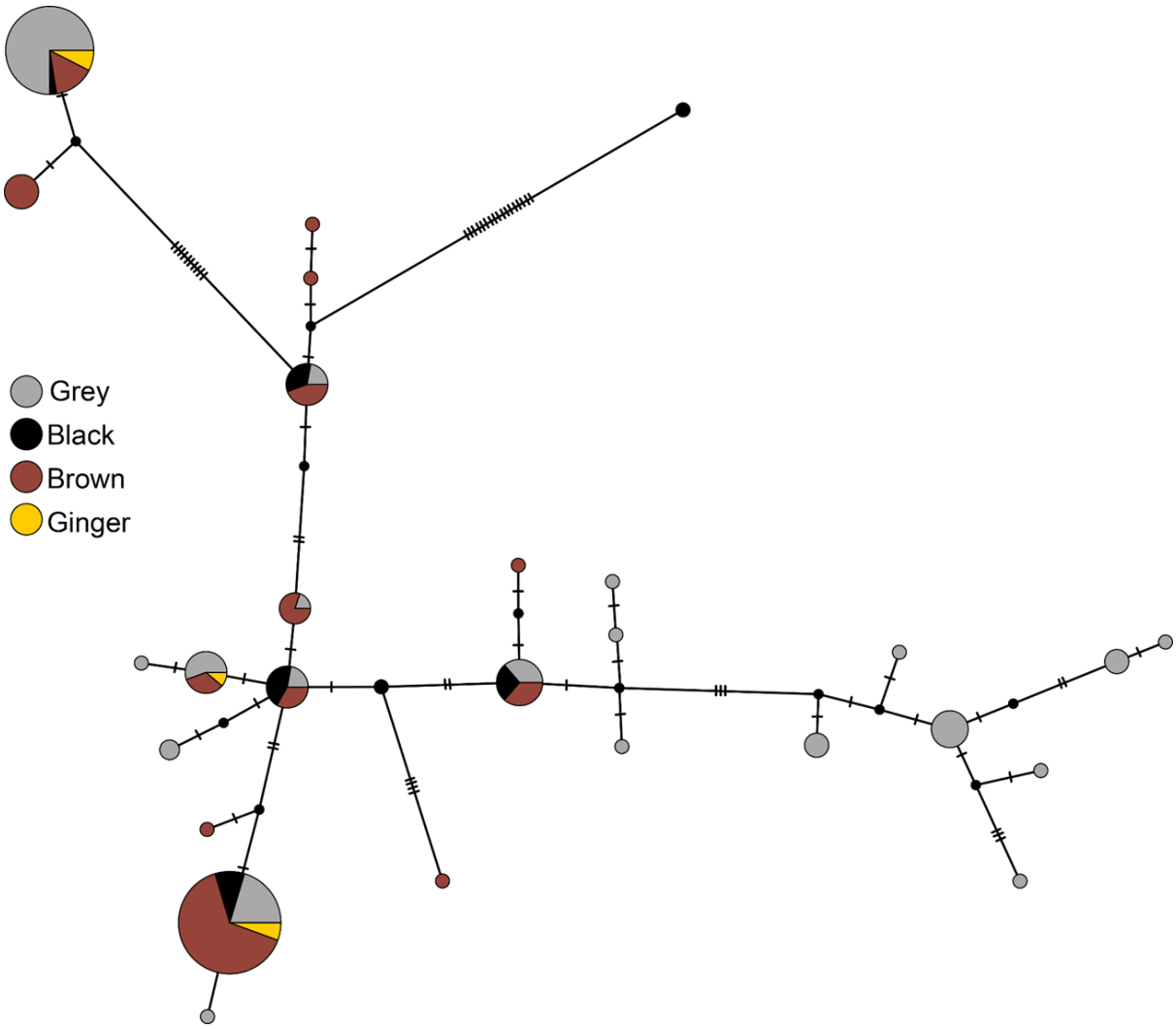

Figure 2.8: Mitochondrial haplotype diversity for possum fur colour using a sequence dataset of 154 individuals. The black node on the top right with 18 nucleotide base differences represents a Tasmanian individual (outgroup). This network differs from 2.7 in that only a subset of the total samples were used for fur colour analysis.

Only a subset of the mitochondrial haplotype sequence dataset was used to identify the coat colour diversity as information about the fur colour was only available for 154 individuals. Figure 2.8 indicates many different haplotypes and a non-uniform distribution of fur colours among the many haplotypes i.e., the different coloured individuals shared common haplotypes with most individuals in the sample set having grey coats. This is indicative of distribution of fur colour within New Zealand.

The grey colour morphs have the largest presence, followed by brown, black and ginger according to my dataset. One can infer from the haplotype network and the information in section 1.1.1 (Figure 1.1) that there is no correlation between the introduction history (fur colour-based introduction) and the current distribution of possums, indicating extensive mixing and post-introduction artificial translocations. The black node on the top right which is most removed from all the other samples (maximum number of nucleotide base differences) is an individual from Tasmania.

2.3.3 Phylogenetic Analysis

The evolutionary relationships among individuals of Australia and New Zealand were inferred with a phylogenetic tree (Figure 2.9). Brushtail possum (T. vulpecula) individuals were brought to New Zealand from Western Australia and Tasmania (Section 1.1.1). However, there is a great deal of mtDNA diversity in Western Australia not represented in my New Zealand samples.

The T. vulpecula mtDNA variation forms three major lineages. One lineage is observed in Northern Australia in both T. vulpecula and T. arnhemensis specimens. Another lineage is seen in possums from three western Australian locations. Individuals from Canberra (Australian Capital Territory) and Tasmania have haplotypes that are part of the only clade that is observed in New Zealand possum populations.

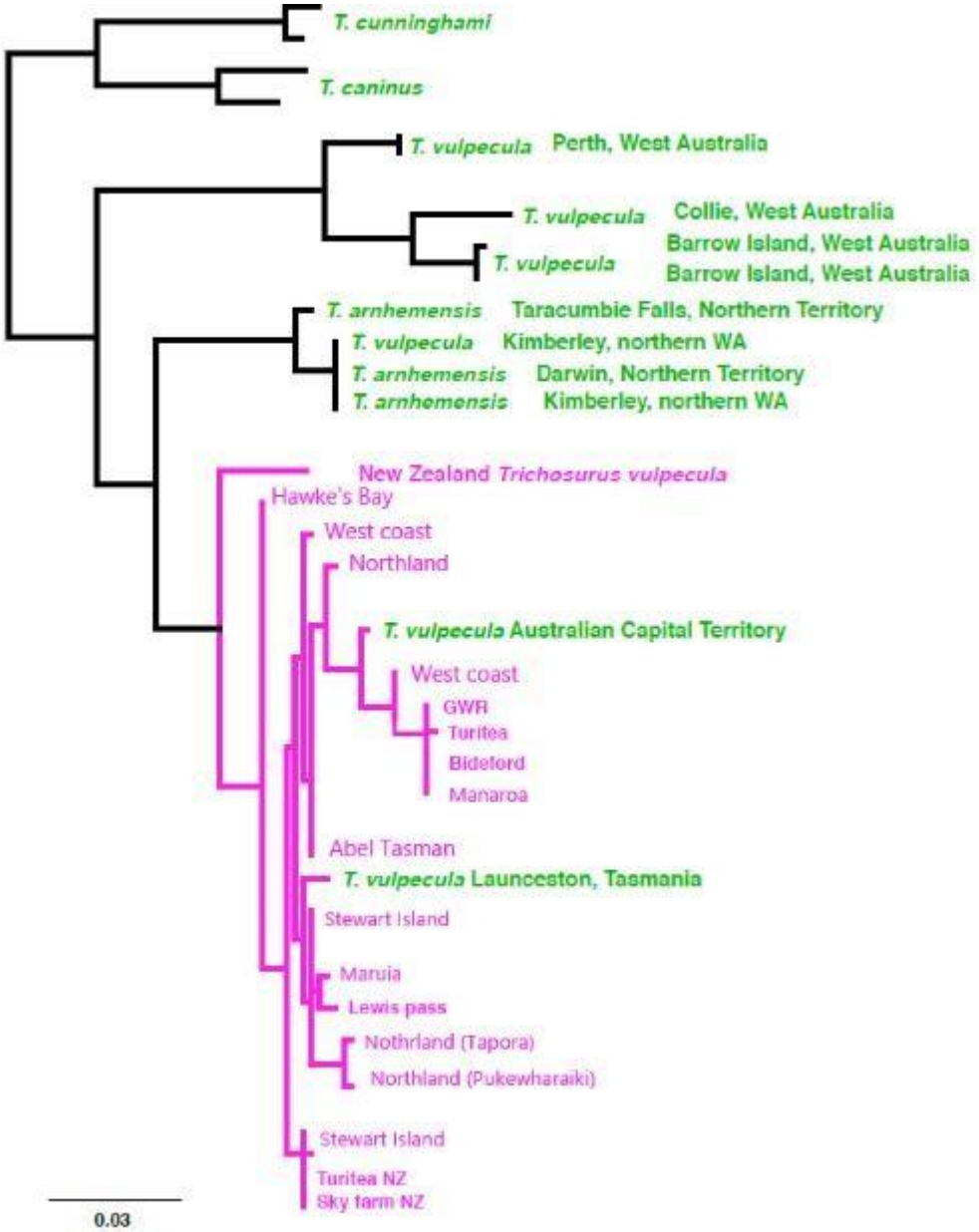

Figure 2.9: Phylogenetic tree constructed using Maximum likelihood with mtDNA (control-region) from brushtail possums. The green labels represent Australian samples and the pink labels represent New Zealand samples.

2.4 Discussion

I have documented mtDNA haplotype variation from brushtail possums collected from all over New Zealand for the first time. Although many source populations in Australia were used to establish the current New Zealand population, I detected representatives of only one of three distinctive mtDNA lineages from Australian populations of brushtail possums. However, fairly high haplotype diversity was observed within New Zealand, with 30 haplotypes recorded.

Population samples with higher haplotype diversities indicate that individuals were probably introduced from many different source populations, bringing with them variable haplotypes. Due to the quantity of individuals being low (small founding number), the nucleotide diversity is also low (Avise, 2000). This is seen in the case of the Pukewharaiki, Tapora and Wairongomai population samples. Increased gene flow between populations could also be the cause of higher haplotype diversities. There is little evidence population structure on a regional level, indicating that there has been admixture between populations with no specific correlation to their geographical location.

Because of the many introductions to New Zealand, their rapid expansion and dispersal abilities, I expected mixing of source populations would result in little genetic structure in New Zealand. Although there was sharing of common haplotypes among population samples, the presence of rare haplotypes resulted in significant differentiation of population samples across New Zealand. The colonisation history can explain regional variation. Small sample sizes of introduction could exhibit random effects (genetic drift). However, substantial differences in haplotype frequencies could also be due to, in case of New Zealand possums, selection for fur colour or management efforts (Takahata & Nei, 1990). When populations could not be differentiated, this was due to the absence of haplotype variation. The consequences of recent management in New Zealand (killing possums) is local genetic bottlenecks. Low nucleotide diversity and low haplotype diversity might indicate that a population has recently undergone a short-term bottleneck effect causing the lowered haplotype diversity. For example this might explain the Manaroa and Bideford population samples, with low diversity relative to the other samples, which could be an effect of recent management efforts, where majority of the individuals in a particular location are removed (along with their haplotypes) from the population (Grant & Bowen, 1998). Population samples such as Kahurangi, Bideford and Skyfarm exhibit overall lowered nucleotide as well as haplotype diversities. My evidence clearly shows that only a small portion of the genetic variants have been introduced from Australia to New Zealand. However, New Zealand possum populations are not all genetically identical, suggesting that colonisation history and/or barriers to dispersal are preventing complete mixing within New Zealand.

Mitochondrial DNA is a great tool but represents a single locus. In the next chapter, I further explore population structure with a multi locus approach.

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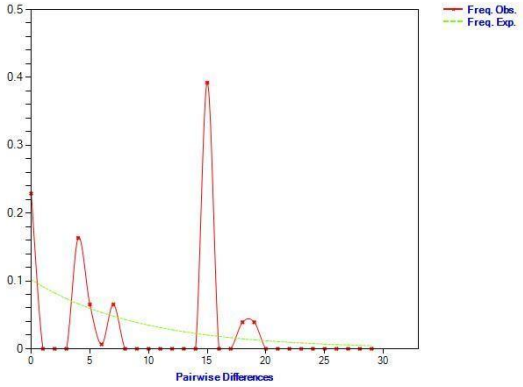
2.6 Appendix

Appendix 1: DNA polymorphism tests for 209 sequences run in DnaSP v 6.12.01.

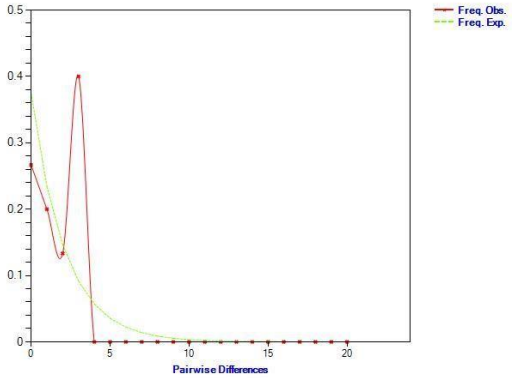
Location	Number of sequences used	Number of pairwise comparisons	Number of polymorphic sites	Average number of differences between sequences	Θw per sequence	Number of haplotypes
West Coast	18	153	21	8.8	6.11	5
Wairongomai	6	15	3	1.6	1.32	3
Manaroa	36	630	19	8.4	4.6	3
Gisborne	10	45	8	3.3	2.8	4
Tapora	11	55	8	2.6	2.75	5
Hawke's Bay	14	91	12	3.8	3.78	4
GWR	28	378	19	2.3	4.88	3
Kahurangi	8	28	0	0	0	1
Abel Tasman	12	66	11	2.1	3.64	4
Pukewharaiki	13	78	15	5	4.84	9
Stewart Island	20	190	30	8.5	8.45	4
Bideford	16	120	1	0.3	0.3	2
Skyfarm	7	21	7	2	2.85	2
Turitea	10	45	21	7.4	7.42	3

Appendix 2: Mismatch Distribution for 13 populations showing the distribution of observed pairwise nucleotide site differences (x-axis) against frequency (y-axis). The red lines with dots represent the observed frequencies of pairwise differences and the green line shows the expected values. On account of haplotype and nucleotide diversity being 0, no graph could be produced for the Kahurangi population.

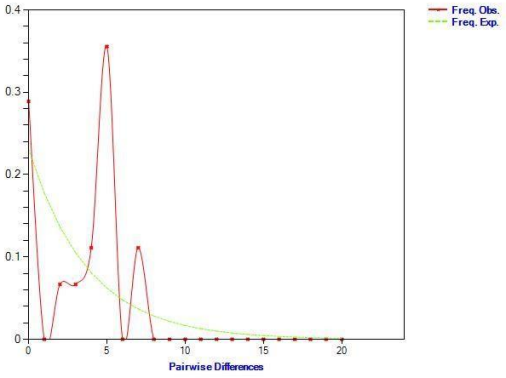
West Coast (WES)



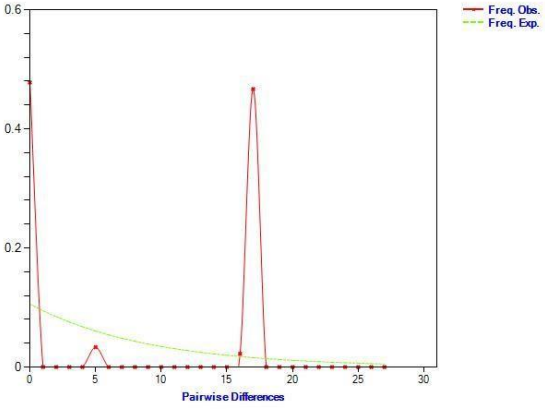
Wairongomai (WAI)



Manaroa (MAN)

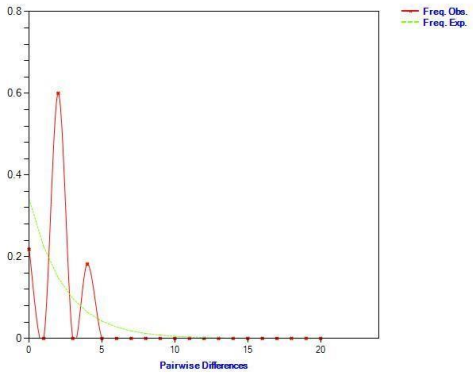


Gisborne (GIS)

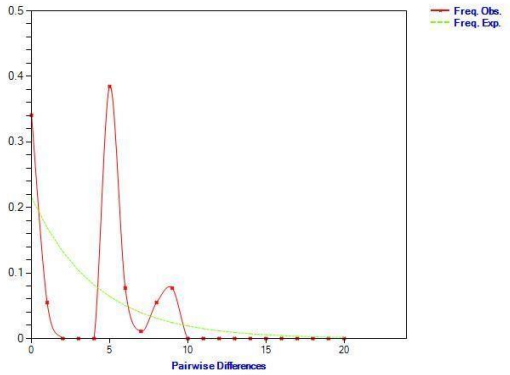


Tapora (TAP)

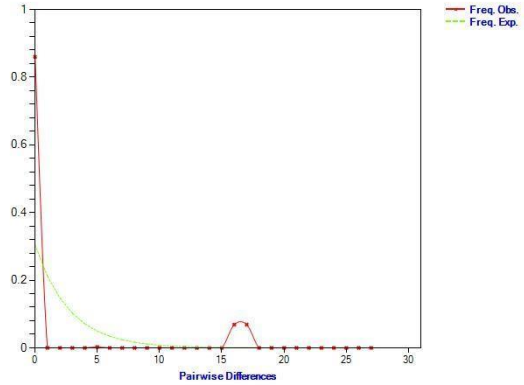
Kokomoka (KOK)



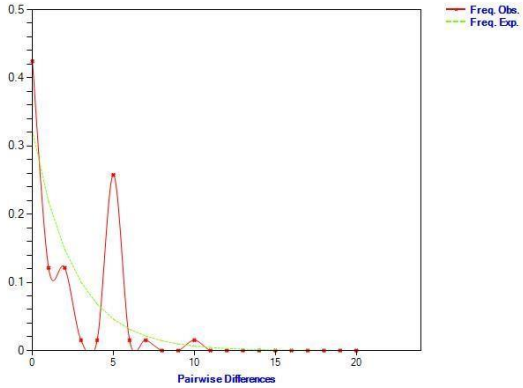
GWR



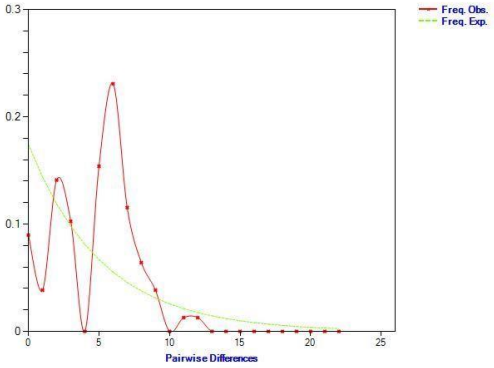
Abel Tasman (ABE)



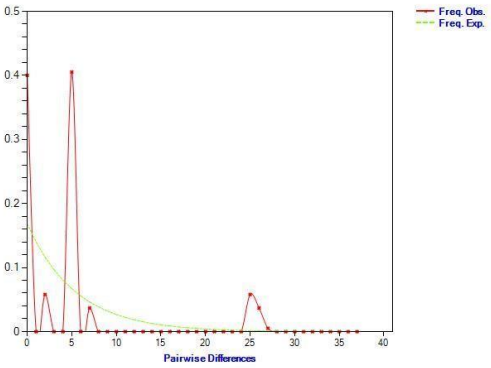
Pukewharaiki (PUK)



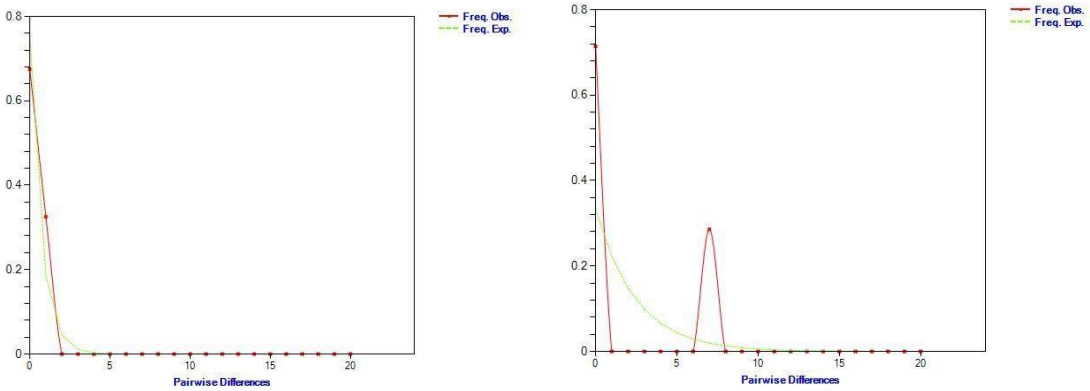
Stewart Island (STE)



Bideford (BID)



Skyfarm (SKY)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Freq. Exp.
Pairwise Differences
Freq. Obs.
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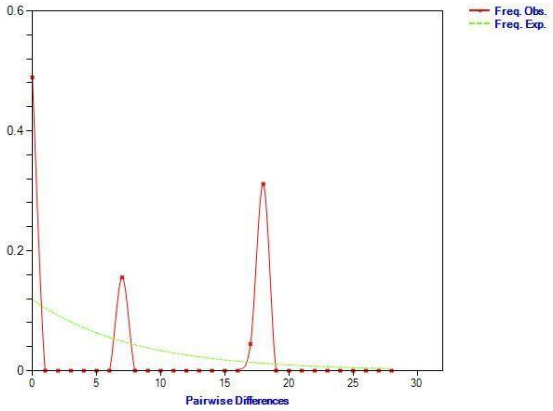
Pairwise Differences

Turitea (TUR)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Pairwise Differences

Chapter 3. Microsatellite Genotype Diversity

3.1 Introduction

Microsatellites are the marker of choice in many population genetic studies of non-model organisms (Sunnucks, 2000). Single copy nuclear microsatellite loci allow species-specific surveys of genetic variation among population samples using the power of the polymerase chain reaction to draw data from a wide variety of tissue types and conditions. Nuclear markers such as microsatellites aid representation of genetic diversity in the population via length polymorphism (Wan, Wu, Fujihara, & Fang, 2004). Microsatellites are short tandem repeat nucleotide motifs that are most useful when many alleles are present within populations, allowing relative measures of genetic diversity and inferences of population structure (Wan et al., 2004). In recent years, traditional population genetic tools have been supplemented by Bayesian modelling tools that allow the naïve estimation of clusters of genetic variants and assignment of individual genotypes to clusters. Bayesian inferences to estimate population gene frequencies can tell us about the origin of an individual in it; these predictions, in this study, are based on previously recorded information with respect to patterns of introduction and establishment of possum populations (Beaumont & Rannala, 2004).

Research in the field of conservation genetics has mostly focussed on endangered species protection, for example, studying population diversity and connectivity between Bengal tiger (Panthera tigris tigris) populations in India (Joshi, Vaidyanathan, Mondol, Edgaonkar, & Ramakrishnan, 2013) or examining allelic differences between two populations of Gila topminnow (Poeciliopsis occidentalis), an endangered fish species in Arizona (Vrijenhoek, Douglas, & Meffe, 1985). Research on invasive species uses similar concepts and methods in understanding the invasive potential of a species introduced to a non-native environment (Suarez, Holway, & Tsutsui, 2008). Population genetic structure of Norway rats (Rattus norvegicus) in France helped estimate gene flow rates between populations using microsatellite markers, thus identifying management units for eradication (Abdelkrim, Pascal, Calmet, & Samadi, 2005). Comparably, a study on feral pigs (Sus scrofa)- invasive mammals introduced to Australia- examined population structure and genetic connectivity; inferring illegal movement of these feral pigs which could affect attempts to control populations (Spencer & Hampton, 2005).

Data generation in population genetics has predominantly entailed the use of molecular markers such as microsatellites, which when analysed over many geographical locations (depending on the scale of study), provides us with important information on the spatial

population structure of the species (Manel, Schwartz, Luikart, & Taberlet, 2003). Successful dispersal of individuals from one population to another results in gene flow that reduces population differentiation but helps maintain genetic diversity within species (Slatkin, 1987). Our estimates of geographic structure of natural populations will be limited by the data generated, the uniformity of sampling, the number of samples in each population as well as the temporal scale of the study (Richardson et al., 2016). Tests for Isolation by distance (IBD) and genetic discontinuities between populations are the most common methods to visualize spatial structure of the study species, which are well supported when done using individuals as the operational unit of the study (Manel et al., 2003). The Bayesian clustering method that follows data generation categorizes the individuals into inferred populations/ genotypic clusters that will inform us of the genetic similarity or dissimilarity between populations, which is an indicator of the gene flow that occurs between them (Adams, 2013). In this study, the populations differ from each other in location, so when populations are more similar, we infer greater gene flow. Gene flow could be from the recent movement of individuals between these locations or could result from the relatedness of founding individuals during the translocations and establishment of populations in New Zealand. In contrast, geographically isolated populations without gene flow will be genetically differentiated from one another (Hutchison & Templeton, 1999).

I examined genetic variation in order to determine whether the population genetic structure of possums across New Zealand reflect their introduction histories from Australia. However, subsequent population management (translocations & killing) of possums in New Zealand might have resulted in genetic uniformity across New Zealand where mixing of individuals from different populations could have a homogenizing effect on their genetic diversity or significantly reducing number of possums by killing could alter gene frequencies, thereby reducing diversity. I surveyed microsatellite genotypes in population samples of possums across the country. A single successful introduction to New Zealand with subsequent range expansion, or multiple introductions of individuals sharing the same genotype would be expected to result in panmixia– lack of population structure (as illustrated in Figure 3.1a). However, separate introductions of genetically distinct individuals to geographically distant locations would result in strong spatial structure if gene flow since establishment was limited (Fig. 3.1b). A combination of distinct populations with mixing is possible if gene flow within New Zealand has been extensive (Fig. 3.1c).

The establishment and increase in Brushtail Possum (Trichosurus vulpecula) numbers over the last century is a clear mark of the generalist nature of this species (PE Cowan, 1990).Their introduction from Australia and their spread across New Zealand thereafter has likely caused some changes in the genetic composition, behaviour and morphology of the species. Section

1.1.3 which focuses on dispersal of possums, along with records of artificial translocations (Pracy, 1974), forms the basis of our hypothesis wherein we expect there to have been sufficient movement of possums (gene flow) between locations to have a homogenizing effect on genetic diversity of the species in New Zealand.

The aspect of spatial structure is important in understanding the ecology of the species with respect to management. Targeting possums in New Zealand is currently very specific to areas surrounding dairy farms and pasture lands where cattle are most vulnerable to contracting Bovine Tuberculosis (Tb) (OSPRI, 2018). Comparing possum population structure across the many regions, especially those situated closer to the next population, improves the understanding of whether a group of these populations need to be treated as individual populations (as they are now) or a group of populations to be treated as a single management unit. This is important in terms of effective possum control as untreated but connected areas could be the cause of possums moving in from near-by locations and establishing new populations (vacuum effect) (Efford et al., 2000).

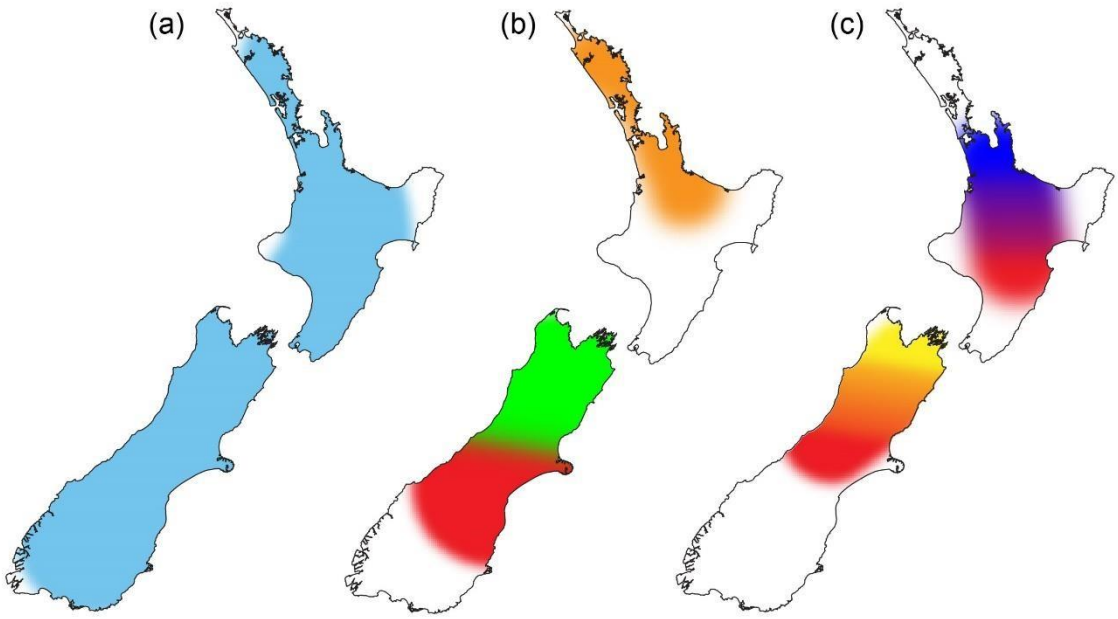


Figure 3.1. Three contrasting patterns of genetic structure arise from three hypotheses of introduction history and gene flow of brushtail possums in New Zealand. 3.1(a) Multiple introductions with similar genotypes resulting in panmictic populations 3.1(b) Genetically and geographically distant populations with limited gene flow. 3.1(c) Genetically and geographically distant populations with extensive gene flow.

3.2 Materials and Methods

3.2.1 Sample Collection

Samples were collected from 19 locations from across New Zealand (Table 3.1; Figure 3.2) by my collaborators: Landcare Research New Zealand (2003), Wellington Regional Council pest control staff (2017-2018) and private owners (2014-2018). Additionally, I analysed genotyped data from a previous study on possum population structure (Taylor et al., 2004) (Figure 3.10) as a majority of the samples used in this study were obtained in 2003. The results of both studies are only comparable at a rudimentary level, as the methods used for data generation differ. I included samples from individuals collected more recently (2014, 2018), to possibly estimate temporal differences. However, there is a possibility that due to the difference in sampling time, I could not account for potential management operations that could affect the results obtained. Some population samples are small (n=1 - 6) although indicative of the spatial pattern of genotypic distribution of possums from these regions, they are removed from some downstream analyses.

Table 3.1: Locations, size and year of sampling for brushtail possums (*Trichosurus vulpecula)* in New Zealand.

Sample location	Region	Sample size	Year of sampling
Fox Valley	West Coast	16	2003
Haast Plains	West coast	10	2003
Stewart Island	Southland	20	2003
Bideford	Wellington	30	2017
Bideford	Wellington	20	2016
Catlins	Southland	1	2017
Taupo Bay	Northland	1	2017
Awaroa Abel Tasman	Tasman	1	2018
Abel Tasman	Tasman	16	2003
Pukewharaiki forest	Northland	16	2003
Kokomoka	Hawke's Bay	16	2003
Whangara	Gisborne	16	2003
Tapora	Northland	16	2003
Aropoaonui	Hawke's Bay	16	2003
Purua	Northland	16	2003
Manaroa	Marlborough	40	2016
Maruia	West Coast	1	2016
Lewis Pass	Canterbury	1	2016
Skyfarm	Tasman	10	2016
Turitea	Manawatu	2, 4, 6	2015, 2016, 2018

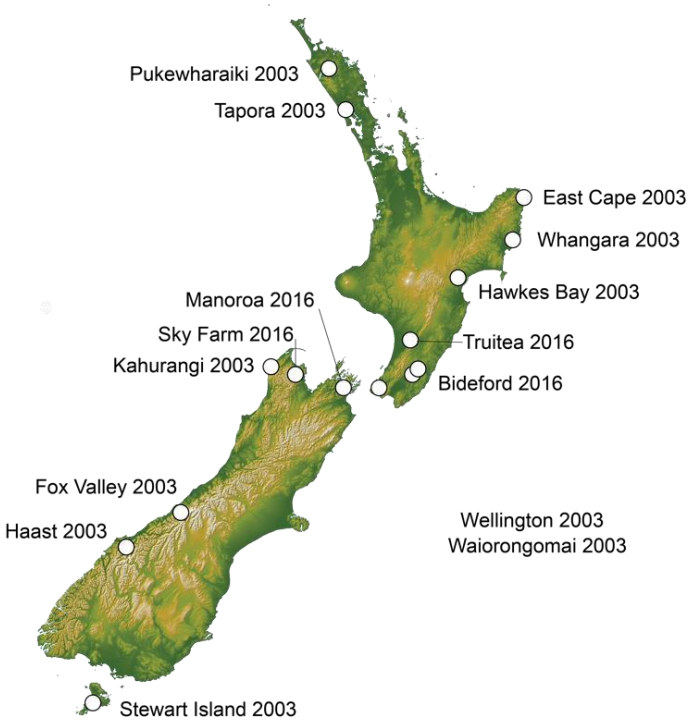


Figure 3.2: Location and year of sampling for brushtail possums (*Trichosurus vulpecula*) in New Zealand

3.2.2 Laboratory methods

I used nine microsatellite primer pairs that were developed previously (Taylor & Cooper, 1998; Taylor et al., 2004). I used two PCR multiplexes with four loci in each (M1: Tv_16, Tv_58, Tv_53 and Tv_19 and M2: Tv_64, Tv_PnMs, Tv_M1 and Tv_14), and locus Tv_27 separately, to amplify the microsatellite loci that were labelled with fluorophores FAM™, VIC™ and TAMRA™ (Table 3.2). PCR reactions consisted of 2µl of Thermopol® buffer, 0.4µl of dNTP, 0.1 µl of Taq DNA polymerase, 0.5 µl of forward and reverse primers made up to a volume of 10 µl by adding water. No additional $MgCl^{++}$ was added to the mixture as adequate amounts were already present in the reaction buffer. Thermal cycling conditions for microsatellite amplification had an initial denaturation step for 4 minutes at 95˚C, followed by 40 cycles of 30 seconds at 94˚C, 45 seconds of 60˚C, and 45 seconds of 72˚C and finishing the reaction with a final extension step for 10 minutes at 72˚C. To test if the procedure worked, I ran the PCR product on an electrophoresis gel with SYBR Safe gel stain. The amplified microsatellite PCR products were then sent to Macrogen Inc. for fragment analysis and identified amplification products of suitable size. Position of the alleles on the locus were determined using the software microsat plugin with Geneious 11.1.4. I had 310 samples in total, but due to non-amplification at four or more loci, 35 samples were removed from this study. The range of

microsatellite loci used in previous research (Table 3.3), of which I amplified a subset of nine commonly used loci (Table 3.2) in order to maximize overlap with data from the other studies.

Table 3.2: Microsatellite primers developed for brushtail possums and used in this study. F:

Locus name	Primer template	Total number of alleles in 19 populations	Allele size range*	Fluorophores
Tv_16	F: GAGGCTACCATTAGACGCAA R: ACCCAAATGAACAGAAAGGC	21	144-146	FAM
Tv_19	F: CCTCCTCCCCATCCTTCCTG R: GTTCAATTGCAGGGCTATGG	26	254-294	VIC
Tv_58	F: GCACCCAAGGACCCCCAAGA R: CCATATCACAGTGCTTGGCG	23	124-168	VIC
Tv_53	F: GGGAGTAGTTGTCTGAGTTCCC R: CCCTGGAGTTTGACAACCTG	35	222-272	FAM
Tv_64	F: AGGGAGACTGAGTGCGTTTG R: AGACAGGAAAATTTGTGCCC	33	138-199	FAM
Tv_PnMs	F: CCACCCCAATTAGATTAGCTC R: GGATGGTTTGTGACAATTTGC	22	220-251	FAM
Tv_M1	F: GACCACAACCTGGGTCTAACCAAC R:CATGACACCTGGGCACTCAGGACT	19	224-252	TAMRA
Tv_27	F: AGTGGAACCACATGTCAGGGC R: GGACTGAAATGACTGCACAAC	26	163-193	TAMRA

Forward Primer R: Reverse Primer * As given by Adams et al.(2013).

Table 3.3: Microsatellite markers used in previous studies to study genetic diversity in Brushtail

Referenes	Tv_16	Tv_19	Tv_27	Tv_53	Tv_54	Tv_58	Tv_64	Tv_M1	Tv_14	Tv_5.64	Tv_PnMS	Tv_38.1
Adams 2013	1	1	1	1	1	1	1	1	1	1	1	1
Clinchy et al. 2004	1	1	1	1	0	1	1	0	0	0	0	0
DeGabriel et al. 2014	1	1	1	1	0	1	1	0	0	0	0	0
Duenas et al. 2014	1	1	1	1	1	1	0	1	0	1	0	0
Sarre et al. 2014	1	1	0	1	0	1	1	1	0	1	1	1
Taylor et al. 2004	1	1	1	0	0	1	1	0	0	0	0	0
Taylor et al. 2000	1	1	1	1	0	1	1	0	0	0	0	0
Blyton 2012	1	1	1	0	0	1	1	1	1	0	0	0
Total	**8**	**8**	**7**	**6**	**2**	**8**	**7**	**4**	**2**	**3**	**2**	**2**

Possums. 1: Markers were used in the study. 0: Markers were not used in the study.

3.2.3. Allelic variation

Uncertainly in allele calls for one locus (Tv_14) led me to test for genotypic errors resulting from null alleles, stuttering and long allelic dropouts using the software MicroChecker 2.2.3 (Cowled et al., 2008). I used two population samples - Kokomoka (n=16) and Manaroa (n=40) as indicators for these errors.

Levels of polymorphism within each population sample were summarised with average number of alleles per locus and observed heterozygosity levels. I tested my population samples for evidence of deviations from Hardy-Weinberg expectations (for nine microsatellite loci using the software Genepop on the web). Running Hardy-Weinberg tests for a set of loci, can be used to throw light on the fundamental biological processes such as inbreeding and random mating (Waples, 2014). Population samples from GWR (n=30), Hawke's Bay (Kokomoka and Aropoaonui) (n=32), Fox Valley (n=26) and Manaroa (n=40) were used for all tests run on this software as they had the largest sample size. Deviations from Hardy-Weinberg Equilibrium (Fisher's method) were tested using 100 batches of 1000 iterations. Furthermore, heterozygote frequencies were used to detect the presence of null alleles at each locus.

Linkage disequilibrium (LD) between a pair of loci is indicated when there is physical linkage between two loci, i.e., they are in the same region of the genome/chromosome (Taylor et al., 2004). Fisher's method was used to check for genotypic linkage disequilibrium between all pairs of loci in the four test population samples using Genepop
on the web. The test operates with the null hypothesis that the genotypes at one locus are independent of the others (Rousset, 2008).

To estimate genetic differentiation among population samples, a pairwise F_{st} (fixation Index-Statistics) semi-matrix using Slatkin's method was computed between 15 population samples (sample size > 1) using Arlequin 3.5.2.2 (Excoffier & Lischer, 2010). The parameters were set to 100 permutations with a significance level of 0.05.

3.2.4 Isolation by distance

Gene flow between geographically adjacent populations, leads to genetic similarity of these populations. If New Zealand possum populations have genetic structure that results from their place of origin in Australia, then the New Zealand populations are not expected to show a pattern of Isolation by distance. If gene flow within New Zealand has been extensive, Isolation by distance will not be observed. An IBD test might throw light on the dispersal

tendencies of the population under study- which could be affected by presence of landscape barriers (mountains, rivers) located between sampled populations (Slatkin, 1987; Wright, 1943). Isolation by distance (IBD) tests were conducted between fifteen possum populations (each in a separate location) using the method described in Rousset (2008). The first step in obtaining an IBD plot is developing a semi-matrix of linear F_{st} estimates. This matrix showing pairwise genetic differences is computed using Mantel tests with 1000 permutations to ensure the significance of the estimates using Genepop on the web. The differences are calculated between each population pair, therefore, populations with single individuals were removed from the analysis as they skewed the results, leaving 15 populations. Following this, a semi-matrix of geographic distances between the same population pairs was generated using the software Geographic Distance Matrix Generator V1.2.3. The Global Positioning System (GPS) coordinates for this matrix were obtained from the various collectors during sampling events. However, when there was a region sampled, a single GPS point was used to identify that population. For the final step, I combined information from the two matrices using the Isolode program (Mantel's method) in Genepop to produce data points to be plotted on the IBD graph.

3.2.5 Naïve genotype assignment

I used a naïve modelling approach that assigns individuals in a sample to genetic clusters implemented in Structure 2.3.2 (Pritchard, Stephens, Rosenberg, & Donnelly, 2000). This model-based analyses seeks to cluster individual genotypes to one or more of the inferred populations based on their allele frequencies (Pritchard et al., 2000). The assigned clusters (K) are based on the assumptions that the populations are in Hardy-Weinberg Equilibrium and the loci under analysis are in complete linkage equilibrium, and each cluster identifies as a subset of allele frequencies (Pritchard et al., 2000). The analysis with a model that fits the data the best is used to infer the mostly number of population clusters (optimal K).

For my microsatellite dataset, I used the Bayesian Markov-Chain Monte Carlo (MCMC) method to cluster the individuals into the inferred populations based on the multilocus data. The Burnin period was set to 5000 and the MCMC repeats thereafter were set to 50,000 to increase the accuracy is assigning individuals to the inferred clusters (Porras-Hurtado et al., 2013). An admixture model was used specifying that the pre-defined populations that were based on location sites were not to be used as priors when assigning the individuals to inferred populations. K-values were tested between 1 to 10 for 275 samples and 8 loci with 10 iterations for each K. The K-value that best fits the dataset was calculated using the Evanno method in the software Structure Harvester (Earl, 2012). Inferring K-value can have setbacks in that we cannot be certain of it as it depends on factors like the allele frequencies, sample

size and rates of dispersal, the priors (if chosen) and the modelling assumptions (Pritchard et al., 2000). Considering all these factors, the ideal K-value is chosen, however, it may not be biologically exact i.e., it may not be the true number of source populations. The (delta) K-value was subjected to the middle-step in the downstream analyses where multiple replicate analysis was used to interpret the difference between the various runs i.e., standardize the various replicates that have the same cluster membership coefficient. This is known as label-switching which provides permuted matrices of all replicates as the output from the software CLUMPP1.1.2 (Jakobsson & Rosenberg, 2007). The final step is the visualization of this output which is in form of a bar plot obtained from distruct 1.1 (Figure 3.6).

RStudio was used to conduct the Structure analysis to provide better visualization of the bar plot that categorised individuals into genotypic clusters (Figure 3.8) with the corresponding map showing the proportion of the genotypes in the population in pie format (Figure 3.7).

3.3 Results and Discussion

I genotyped 275 possums for nine microsatellite loci. Locus Tv_14 amplified, but I could not ascertain the size of all alleles as the results were unclear (Figure 3.3). Tv_14 alleles overlap in size with alleles at other loci amplified in the same reaction (Tv_27 and Tv_M1), this may have caused the distortion. In addition, I detected the presence of null alleles at locus Tv_14. Homozygote allele frequencies at locus Tv_14 (Figure 3.4) for Kokomoka and Manaroa populations clearly indicate that the observed homozygote frequencies are much higher than the expected range. An excess of homozygotes at one locus indicates the presence of null alleles as this condition was not seen in other loci. Therefore, I removed this locus from further analyses.

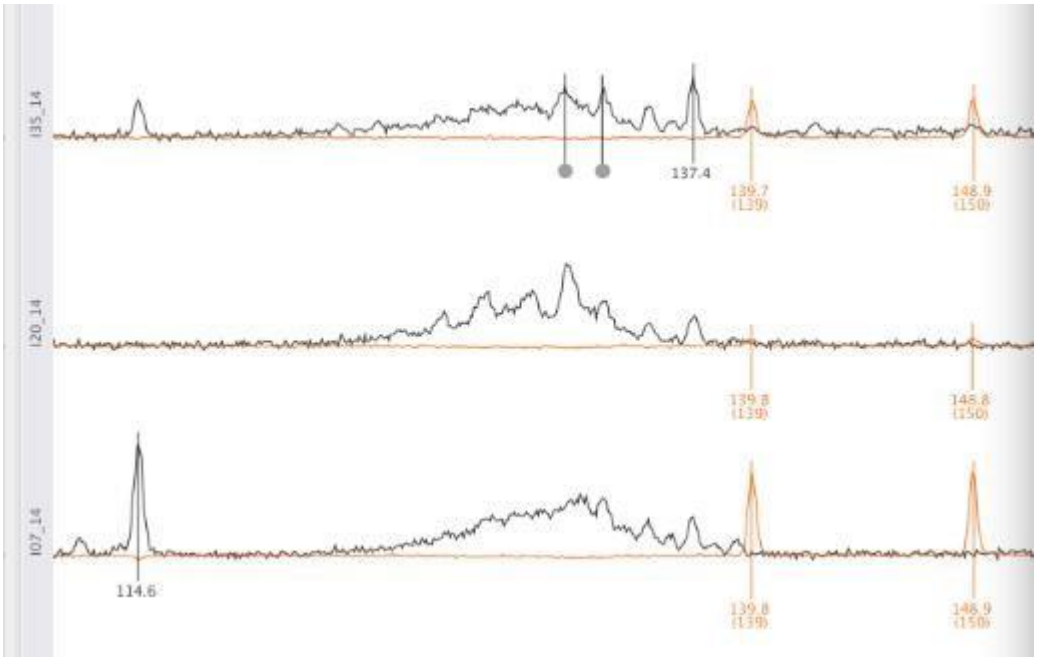


Figure 3.3: Distorted allele peaks for locus Tv_14 in *Geneious.* The allelic range for Tv_14 was 116 to 145 while allelic range for Tv_27 was 128 to 154.

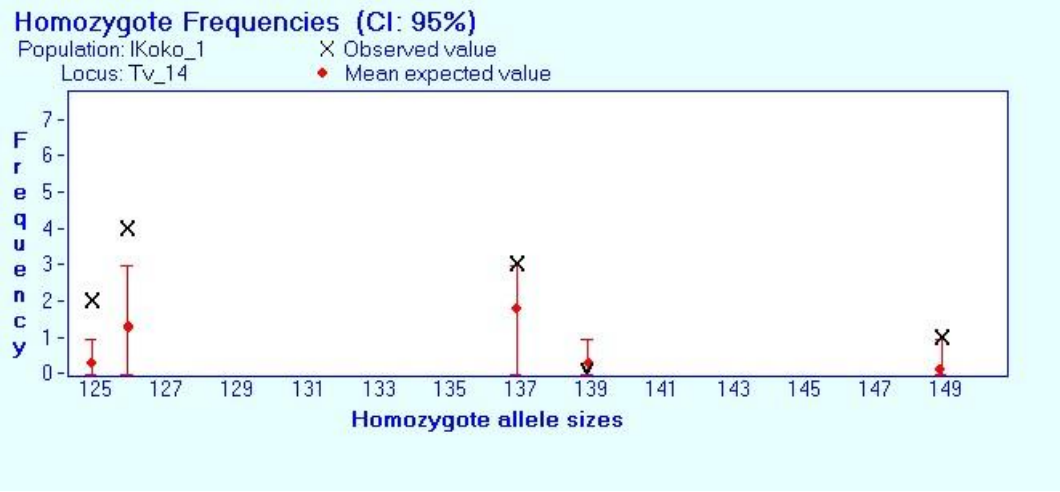


Figure 3.4: Homozygote frequencies as shown in *MicroChecker* for locus Tv_14 with the observed values exceeding the expected range of homozygotes in the Kokomoka population.

3.3.1 Linkage disequilibrium

I found that of all the pairs of loci, Tv_16 and Tv_27 were linked, indicated by p-value = 0, for the Manaroa population (Table 3.4). Linkage disequilibrium test for the other three populations (GWR, Hawke's Bay and Fox Valley) showed similar results. There has been evidence in some previous studies that Tv_27 was linked to locus Tv_16 (Taylor & Cooper, 1998), but has been used in studies that followed (Table 3.3). Additionally, there was an

absence of allele calls for Tv_27 in some populations (Bideford 2016, Sky farm 2016), therefore Tv_27 was taken out of further analysis. Due to the number of pairwise test (n=27) I did not regard $P < 0.02$ as biologically significant (Bonferroni correction).

Table 3.4: Linkage disequilibrium test for the Manaroa population using Fisher's method on Genepop shows Tv_16 and Tv_17 (bold) to be linked; P-value= 0.

Locus Pair	P-value	S.E
Tv_16 &Tv_58	0.828	0.021
Tv_16 & Tv_53	0.188	0.027
Tv_58 & Tv_53	0.074	0.017
Tv_16 & Tv_19	0.034	0.007
Tv_58 & Tv_19	0.833	0.021
Tv_53 & Tv_19	0.275	0.034
Tv_16 & Tv_64	0.705	0.029
Tv_58 & Tv_64	0.321	0.033
Tv_53 & Tv_64	0.769	0.029
Tv_19 & Tv_64	0.192	0.025
Tv_16 & Tv_PnMS	0.265	0.014
Tv_58 & Tv_PnMS	0.531	0.015
Tv_53 & Tv_PnMS	0.752	0.016
Tv_19 & Tv_PnMS	0.801	0.009
Tv_64 & Tv_PnMS	0.967	0.005
Tv_16 & Tv_M1	0.041	0.013
Tv_58 & Tv_M1	0.999	0.001
Tv_53 & Tv_M1	0.882	0.024
Tv_19 & Tv_M1	0.719	0.033
Tv_64 & Tv_M1	0.249	0.037
Tv_PnMs & Tv_M1	0.485	0.017
Tv_16 & Tv_27	**0**	**0**
Tv_58 & Tv_27	0.226	0.031
Tv_53 & Tv_27	0.171	0.032
Tv_19 & Tv_27	0.201	0.025
Tv_64 & Tv_27	0.822	0.031
Tv_PnMS & Tv_27	0.209	0.015
Tv_M1 & Tv_27	0.011	0.009

3.3.2 Allelic variation analyses

Each of the seven microsatellite loci contain between 2 and 13 alleles, with average allele number highest (9.25) in the largest populations sample (GWR; Table 3.5). Total number of alleles per locus per population sample is reported in Appendix 2. The average gene diversity represented by the Garza- Williamson Index (G-W Stat) shows Tapora and Turitea to have the highest and lowest gene diversity respectively. However, the overall G-W range is close to 0, indicating that populations have been through a bottleneck effect in past as opposed to stationary populations (closer to 1) (Excoffier & Lischer, 2010).

Observed heterozygosity was either as expected or lower than expected in each population sample. Hardy-Weinberg tests for seven loci in 15 populations revealed no significant deviations from random mating. Mean observed heterozygosity (H_o) for all populations were lower than mean expected heterozygosity (H_e). Fox Valley, Abel Tasman, Bideford and Turitea populations showed a greater difference between H_o and He. Lowered H_o could also be due to the Wahlund effect- which is a reduction in heterozygosity due to population substructure (Waples, 2014). When a population is subdivided, with slightly different allele frequencies due to mating with neighbours rather than randomly, the overall heterozygosity levels are reduced, even if the subpopulations are individually in Hardy-Weinberg equilibrium (Dharmarajan, Beatty, & Rhodes Jr, 2013). In this case, the reduced H_o may be an effect of population samples representing fairly large geographic areas (Waples, 2014).

Most pairwise comparisons of population samples revealed significant genetic differentiation (pairwise F_{st} semi-matrix; Table 3.6) bolded F_{st} values indicating significant departures from zero). The F_{st} values range from 0.011 to 0.39 with only one population pair showing no genetic differentiation (Kokomoka-Gisborne). Highest F_{st} value of 0.39 was between Skyfarm and Tapora (1,085km apart) followed by an F_{st} value of 0.38 between Stewart Island and Skyfarm.

Table 3.5: Genetic diversity at seven microsatellite loci among 15 populations of Brushtail possums across New Zealand. n: Number of individuals, A: Average number of alleles in the population, H_o: Mean observed heterozygosity, He: Mean expected heterozygosity, and G-W Stat: Garza Williamson Index

Location	n	A	H_o	H_e	G-W Stat
FOX	16	7.13	0.547	0.723	0.16
HAA	10	5.63	0.7	0.718	0.22
STE	20	3.74	0.628	0.648	0.27
GWR	30	9.25	0.746	0.843	0.12
ABE	17	6.87	0.529	0.723	0.15
PUK	16	8.5	0.781	0.828	0.29
KOK	16	6.28	0.678	0.684	0.3
GIS	16	5.86	0.705	0.744	0.14
TAP	16	6.25	0.64	0.646	0.4
ARO	16	6.25	0.718	0.75	0.34
WAI	16	5.5	0.539	0.584	0.38
MAN	40	7.87	0.675	0.737	0.21
BID	20	9	0.591	0.832	0.12
TUR	12	7.13	0.489	0.634	0.07
SKY	10	5.17	0.55	0.66	0.25

Table 3.6: New Zealand population samples of brush tailed possum are genetically differentiated from one another based on seven microsatellite loci using pairwise F_{st} estimates (Slatkin's method) between 15 population samples. Significant F_{st} estimates shown by bolded numbers; non-significant estimates shown in grey.

	FOX	HAA	STE	GWR	ABE	PUK	KOK	GIS	TAP	ARO	WAI	MAN	BID	TUR
HAA	**0.12**													
STE	**0.09**	**0.19**												
GWR	**0.11**	**0.13**	**0.18**											
ABE	**0.08**	**0.12**	**0.15**	**0.11**										
PUK	**0.15**	**0.15**	**0.25**	**0.12**	**0.17**									
KOK	**0.11**	**0.19**	**0.21**	**0.19**	**0.16**	**0.21**								
GIS	**0.07**	**0.18**	**0.19**	**0.16**	**0.14**	**0.17**	0.011							
TAP	**0.27**	**0.25**	**0.36**	**0.23**	**0.29**	**0.12**	**0.32**	**0.29**						
ARO	**0.12**	**0.15**	**0.23**	**0.13**	**0.16**	**0.11**	**0.15**	**0.12**	**0.15**					
WAI	**0.17**	**0.25**	**0.29**	**0.21**	**0.24**	**0.19**	**0.17**	**0.14**	**0.24**	**0.08**				
MAN	**0.16**	**0.2**	**0.21**	**0.15**	**0.16**	**0.19**	**0.27**	**0.24**	**0.29**	**0.21**	**0.28**			
BID	**0.27**	**0.29**	**0.36**	**0.23**	**0.27**	**0.25**	**0.34**	**0.31**	**0.35**	**0.26**	**0.34**	**0.27**		
TUR	**0.21**	**0.24**	**0.29**	**0.19**	**0.22**	**0.23**	**0.27**	**0.25**	**0.34**	**0.23**	**0.32**	**0.23**	**0.12**	
SKY	**0.28**	**0.31**	**0.38**	**0.25**	**0.29**	**0.28**	**0.31**	**0.28**	**0.39**	**0.28**	**0.36**	**0.32**	**0.19**	**0.09**

3.3.3 Isolation by Distance

Possums were introduced from many locations in Australia and released at many NZ sites, therefore I did not expect to detect a population structure within New Zealand that could only arise from a stepping stone model of gene flow among wild populations.

There was no association between genetic and geographic distance for the pairs of populations (Figure 3.5), suggesting no isolation by distance between the populations in my dataset. Genetic differences among populations was detected, but these differences are due to natural gene flow. Population samples from Tapora and Stewart Island differ from one another (maximum genetic distance).

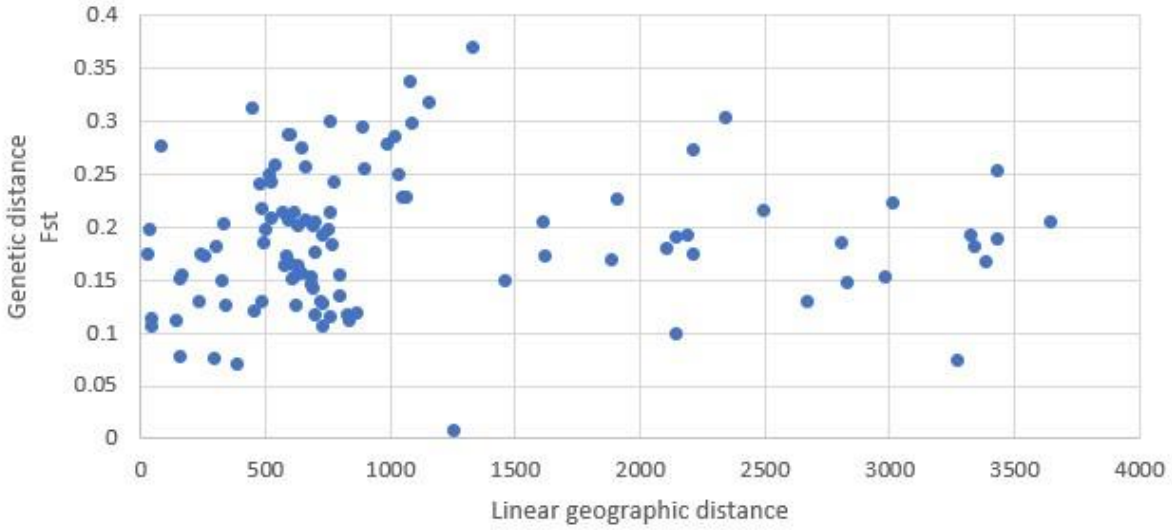


Figure 3.5: Isolation by distance graph showing the genetic distance versus geographic distance between populations. Each dot represents a pair of populations.

The underlying theory behind Isolation by distance is that gene flow between populations is proportional to the geographic distance that separates them (Hardy & Vekemans, 1999). This is generally the case, but we are aware that brushtail possums not only disperse ~10km on average, but also that they were subjected to artificial translocations for a century after their introductions to New Zealand (Pracy, 1974).Therefore I did not expect to detect a signature of isolation by distance.

However, population samples from the South Island did show significant isolation by distance. The North Island population samples of possums were more genetically differentiated, which could be due to translocation history from Australia (and possibly landscape barriers within New Zealand). In contrast, the South Island populations that were geographically closer to one another showed an increased level of genetic similarity than those farther away suggesting gene flow is homogenising adjacent populations (Taylor et al., 2004).

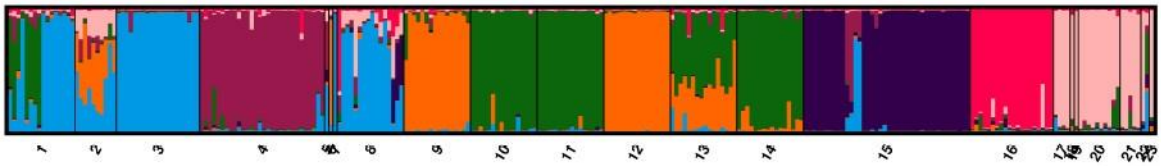
3.3.4 Naïve genotype assignment

I ran 10 iterations for K= 1 to K= 10 inferred populations for the Structure analysis. K=8 (Appendix 1) clusters were identified as the true number of inferred populations for our dataset of 275 individuals in Structure Harvester. A bar plot representing each K-value including the final bar plot (k=8) was created using CLUMPP and distruct (Figure 3.6). Structure Harvester generates a K-value based on the highest likelihood with all the assumptions taking into account (Jakobsson & Rosenberg, 2007). However, in reality, we know that we are just estimating the most likely number of population clusters within our data (Verity & Nichols, 2016) and so the final bar plot for K= 8 is compared with K= 7 and K= 9 plots to observe any changes in population structure.

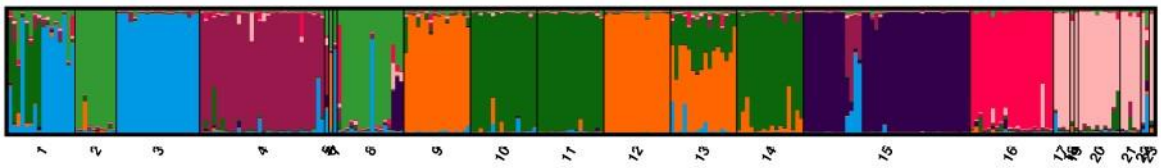
The bar plot divided between 23 population samples (Table 3.7) based on their location as well as year of sampling, to clarify the difference in population structure (Figure 3.8). The inferred optimal fit of model to data has 8 clusters of populations (each in a different colour). The number of inferred genetic clusters (K) indicates that individuals belong to genetically distinct sources. Assignment of each individual is based on their genotype not on their collecting location, some samples from one location are assigned predominantly to one cluster (eg- Kokomoka, Bideford) while some populations are observed to have more admixture (Pukewharaiki, Fox Valley, Manaroa). Assignment plot (K= 8) will be discussed in detail in section 3.3.5.

Here, there is no major difference in population structure between K= 7, K= 8 and K= 9. This comparison was necessary to examine how population structure would vary depending on the number of clusters in the model. The assignment of each individual to a cluster was entirely based on allele calls for the set of 7 microsatellite markers included in this study. In my sample of New Zealand possums, individuals collected from the same location tended to have similar genotypes and therefore were inferred as part of the same genetic clusters. Where samples came from regions in geographical proximity, they often shared predominant genotypic clusters (for example Kokomoka, Whangara). This was not the case with regions that were geographically distant (for example- Kokomoka and Manaroa). Some individual possums had assignment to two clusters with equal probabilities (e.g. Caltins) which normally results from gene flow and some population samples contained individuals that were assigned (with high probability) to different genetic clusters (e.g. Manaroa).

K=7



K=8



K=9

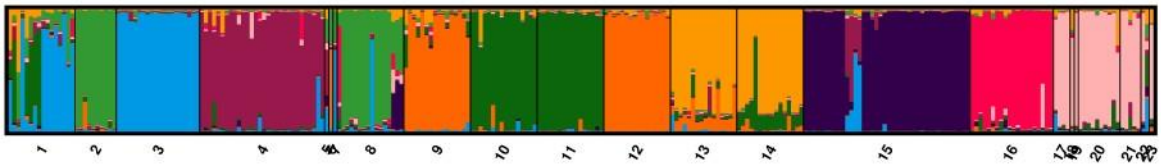


Figure 3.6: Comparison of assignment plots for models with differing number of population clusters (K=7, K=8 and K= 9). Each bar represents an individual, each colour a genotypic cluster. The length of the colours in bar (admixture coefficient) is the assignment probability that the individual's genotype belongs to those clusters. The numbers on the x-axis represent the samples in which the individuals belong (enumerated in table 7).

3.3.5 Population Structure across New Zealand:

I examined the geographic distribution of the eight genetic clusters (identified with Structure) on a map of New Zealand (Figure 3.7) Pie charts are used to represent population samples rather than individual assignments, each represents the proportion of genotypes within that population (size of the pies depended on the sample size). Thus, the proportions of genotypes within the pies are divided in a manner that is not indicative of whether only a few individuals from that sample have been assigned one genotype (in smaller segment) or one individual is assigned multiple genotypes in different proportions (indicated by length of colour in each bar). This discrepancy is resolved in figure 3.8, where the same 8 genotypic clusters are in bar plot format and each individual's genotypic composition can be distinguished.

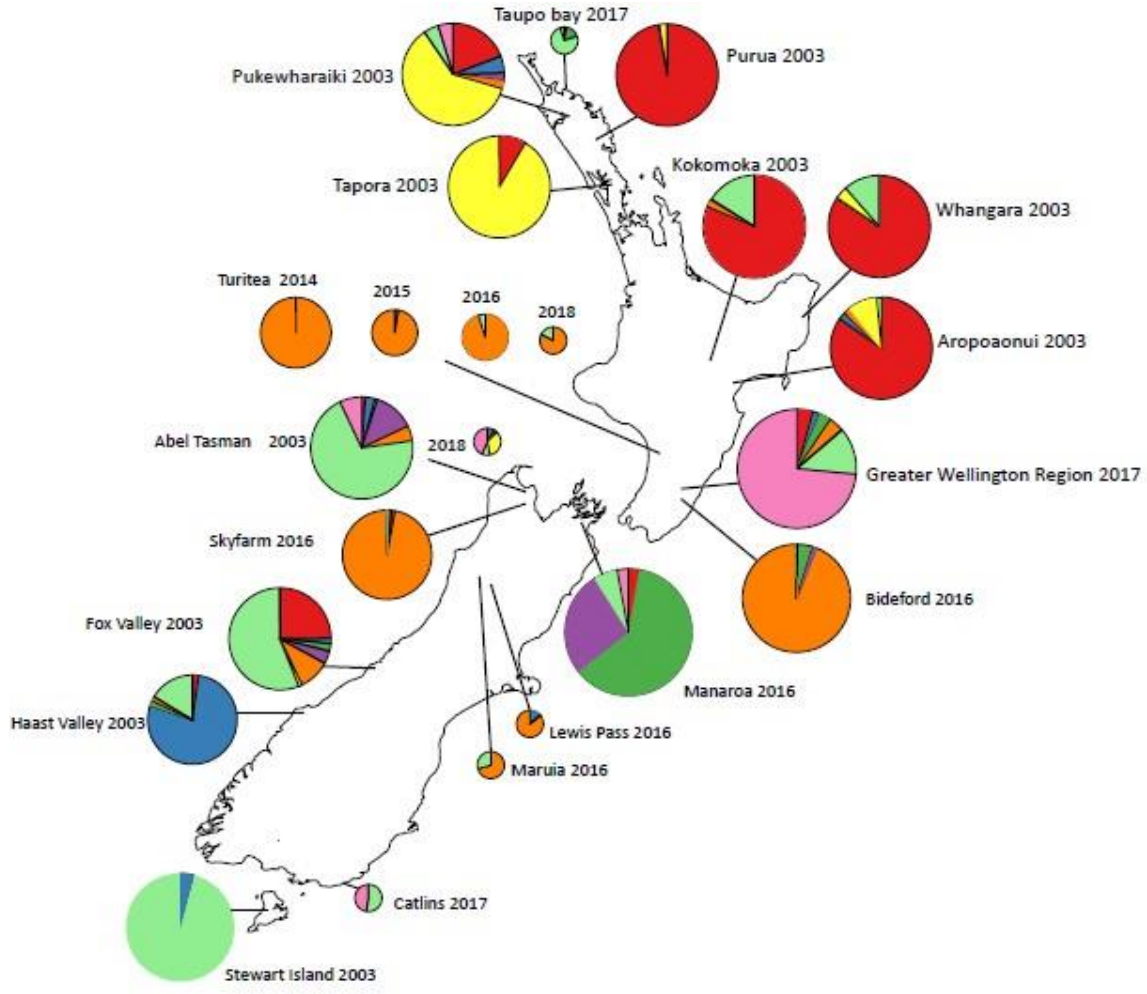


Figure 3.7: Map of sample sites in New Zealand with pie charts showing the proportion of each genotype in that population. Each colour represents a genotype cluster and the size of each circle is dependent on the sample size from that location. Proportion of genotypes can be clearly viewed in the bar plot (generated from the same analysis) shown in Figure 3.8.

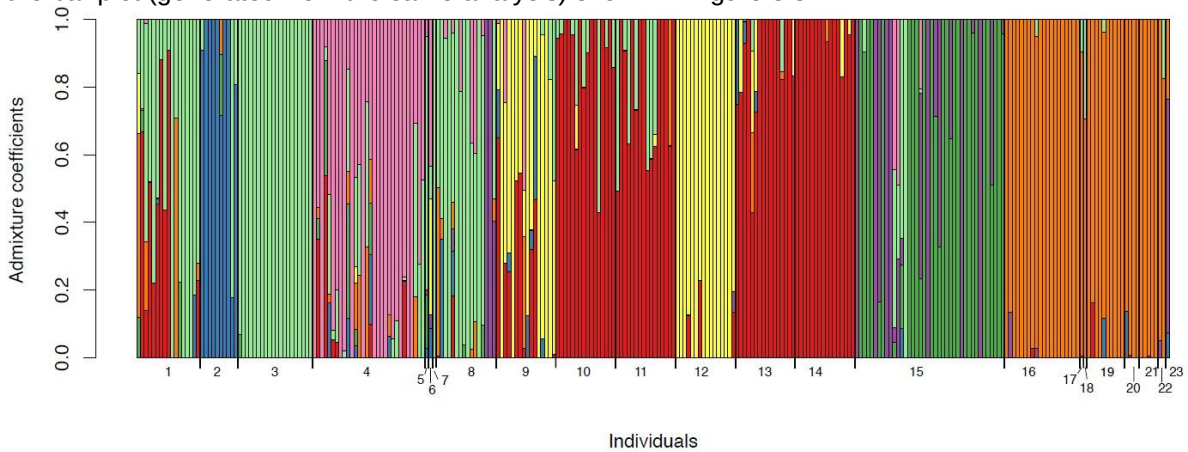


Figure 3.8: Bar plot from Structure analysis in R (K = 8). Each bar represents an individual, each colour a genotypic cluster. The length of the colours (y-axis) in each bar (admixture coefficient) is the assignment probability that the individual's genotype belongs to those clusters. The numbers on the x-axis represent the samples in which the individuals belong (enumerated in table 3.7).

Table 3.7: Populations corresponding to those on the bar plot (Figure 3.8) resulting from the Structure Analysis on R.

Population	Location and year of sampling	Sample Size
1	Fox Valley 2003	16
2	Haast Valley 2003	10
3	Stewart Island 2003	20
4	Greater Wellington Region (GWR) 2017	30
5	Catlins 2017	1
6	Taupo Bay 2018	1
7	Abel Tasman 2018	1
8	Abel Tasman 2003	16
9	Pukewharaiki 2003	16
10	Kokomoka 2003	16
11	Whangara 2003	16
12	Tapora 2003	16
13	Aropoaonui 2003	16
14	Purua 2003	16
15	Manaroa 2016	40
16	Bideford 2016	20
17	Maruia 2016	1
18	Lewis Pass 2016	1
19	Skyfarm 2016	10
20	Turitea 2014	5
21	Turitea 2016	4
22	Turitea 2015	2
23	Turitea 2018	1

The 23 pie charts (Figure 3.7) constitute different populations and year of sampling. Some genotypic clusters are widespread, for example, the light green cluster that is primary in Stewart Island is also the most prevalent cluster in the Fox Valley and Abel Tasman populations of the South Island. This genetic cluster is a small proportion of populations further north in the Whangara, GWR and Kokomoka population samples of the North Island. This is due to some alleles being widespread. However, other clusters such as the yellow group are location-specific (i.e., Pukewharaiki, Tapora and in smaller segments Aropoaonui and Whangara). This specificity of clusters to regions creates a distinct structure of possum populations across New Zealand.

Population structure across Cook Strait:

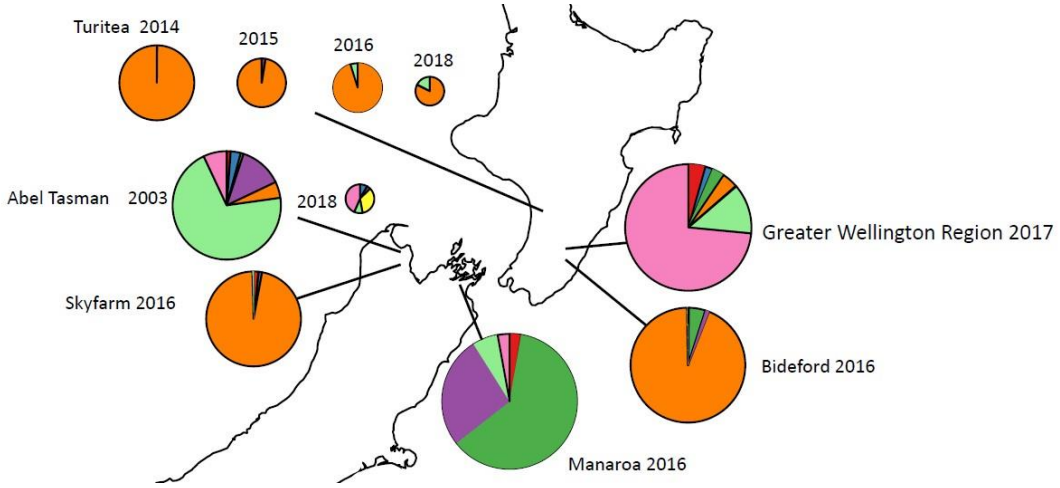


Figure 3.9: Close up of map shown in figure 3.7 focussing on populations across Cook Strait namely Turitea, GWR, Bideford in the North Island and Abel Tasman, Skyfarm and Manaroa in the South Island.

On a finer scale, an unusual pattern of genotype distribution across the Cook Strait is observed (Figure 3.9). GWR (2016) and Bideford (2017) populations, located in close proximity, show stark differences in their genetic composition. Although Skyfarm , Turitea and Bideford share the sample predominant genetic cluster (orange), populations between them (Abel Tasman and Manaroa) show a mix of other genotype clusters. The Turitea population samples, have a similar genotypic composition as Bideford in the North Island and Skyfarm in the South Island. The admixed population of Manaroa (n= 40) and GWR (n= 30), both sampled in 2016, suggest that the Cook Strait could act as a barrier to natural gene flow. However, there is no barrier to explain the difference between GWR and Bideford. Perhaps this structure results from founding effects. The fact that possums were artificially dispersed to different parts of the country for fur trade as well as bounty hunting in the mid1900s (Pracy, 1974) would not result in adjacent populations being so different. Finally, Turitea samples do not show variation in cluster assignment over time, indicative of a pattern of continual occupation within this region.

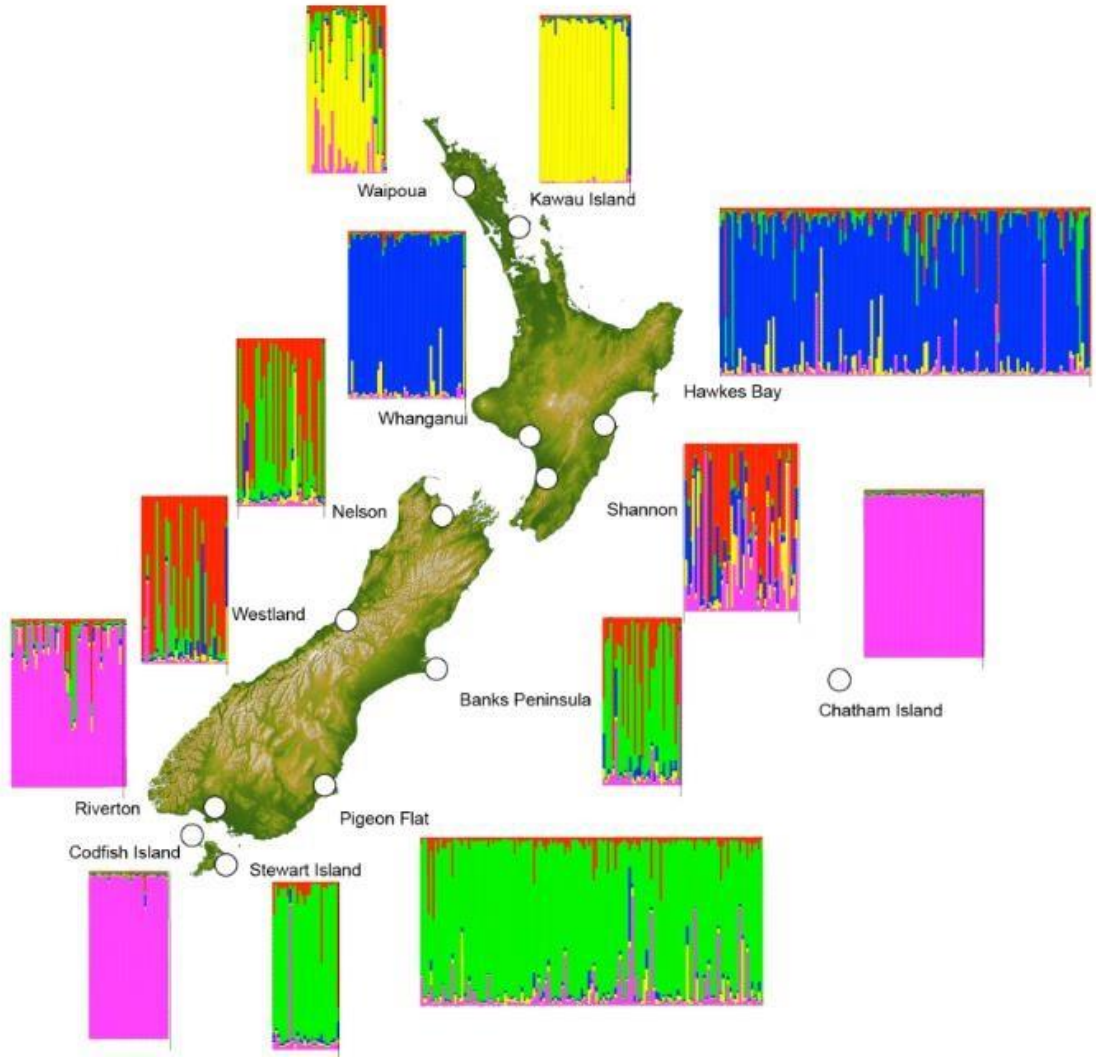


Figure 3.10: Structure plot (K= 5) developed using microsatellite data from Taylor et al. (2004); n= 750

Taylor et al. (2004) previously conducted a large-scale study using five polymorphic microsatellite loci to analyse the genetic structure of 750 possums in New Zealand. Taylor et al. (2004), with their analysis, concluded that Australian samples were found to have higher levels of diversity in comparison to New Zealand, and within the country, North Island populations were more diverse than those of the South Island.

I used the raw data from this study to conduct a Structure analysis, using the same methods and parameters as with my dataset. This analysis was done separate to mine as only five microsatellite loci were used in the Taylor et al. (2004) study; four of which were common with my study (Tv_16, Tv_19, Tv_58 and Tv_64). I used the data (allele calls) to run the Structure analysis and found that K= 5 was the optimal number of clusters when fitting the data to model. Looking at the results of the analysis (Figure 3.10) at a finer scale, samples from Whanganui (~81 km northwest of Turitea) to belong to one predominant cluster shared

with Hawke's Bay samples while Shannon (~30 km southwest of Turitea) to show extensive admixture of genotypes. This led to the inference that there was a high level of variation across New Zealand (potentially beneficial to the establishment of possum populations).

My study shows a similar pattern as the previous study in that the populations of the South Island have common genotypes and similarly, northernmost populations share the major genotypes. The populations located near the Cook Strait show a higher level of admixture between genotypes.

Significant genetic differentiation between most population pairs (F_{st} analysis- section 3.3.2), and Bayesian clustering methods show definite population structure across the country. Considering their complex introduction histories- multiple additions to populations with a small number of individuals, their dispersal tendencies and artificial movement, this level of differentiation among populations is surprising. Even though fractions of the source populations in Australia may have been introduced to New Zealand, the surviving individuals did not remain at such small numbers for a prolonged period of time. This, along with further intermixing of populations, could be the cause of successful establishment, preventing extended population bottlenecks.

I did not find that possums in New Zealand are genetically uniform as might result from multiple introductions of individuals sharing the same genotype with subsequent range expansion. I found genetically distinct individuals restricted to geographically distant locations as expected from their colonisation history, and strong spatial structure as expected if gene flow was limited. Signatures of gene flow are seen in populations and individuals that have low assignment probabilities, and mixed populations, but these are less common than the general pattern of local differentiation, suggesting gene flow is restricted.

3.4 References

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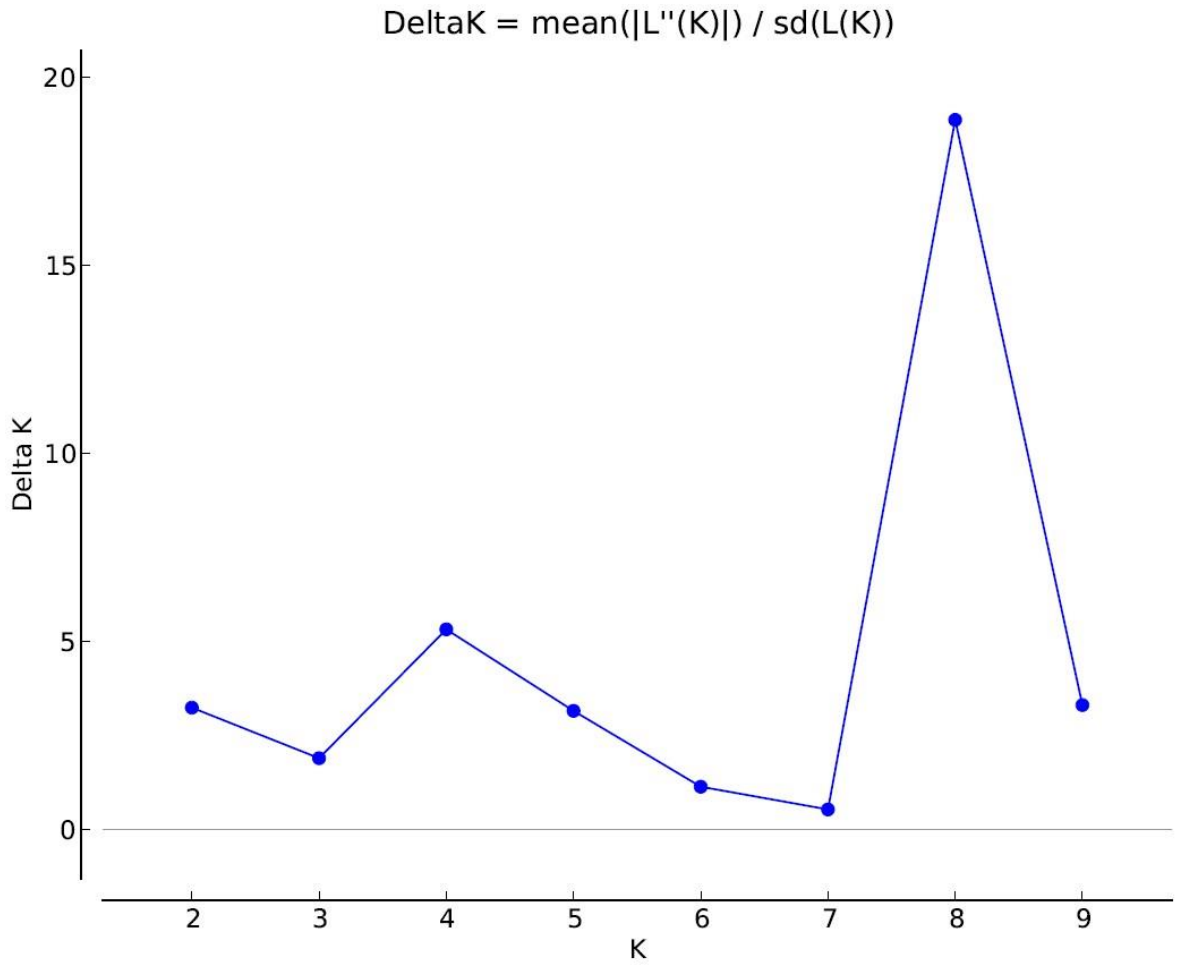
3.5 Appendix

Appendix 1: Using Evanno's method (Earl, 2012) in the program Structure Harvester to identify the true number of inferred populations (K).



Appendix 2: Total number of alleles per locus per population obtained from Arlequin 3.5.2.2.

Locus#	FOX	HAA	STE	GWR	ABE	FOK	KOK	GIS	TAP	ARO	WAI	MAN	BID	TUR	SKY	Mean	s.d.	Tot. number
1	7	5	2	8	4	8	6	5	8	7	4	6	9	6	5	6.000	1.890	22
2	4	6	1	10	6	7	1	1	9	6	7	6	8	11	3	5.733	3.195	24
3	9	5	4	11	7	6	7	9	5	9	7	12	13	10	7	8.067	2.658	36
4	7	6	3	10	6	12	6	5	7	6	5	6	8	11	6	6.933	2.404	27
5	10	6	4	11	12	8	7	5	2	2	2	10	10	9	7	7.000	3.402	34
6	4	4	5	7	5	8	3	3	4	5	4	5	6	5	3	4.733	1.438	21
7	7	4	4	7	9	7	6	6	7	6	5	12	1	2	1	5.600	2.947	20
8	9	9	4	10	6	12	9	8	8	9	10	6	1	3	1	7.000	3.381	27
Mean	7.125	5.625	3.375	9.250	6.875	8.500	5.625	5.250	6.250	6.250	5.500	7.875	7.000	7.125	4.125	6.383	1.559	26.375
s.d.	2.232	1.598	1.302	1.669	2.532	2.268	2.504	2.550	2.375	2.252	2.449	2.949	4.209	3.603	2.475	2.464	0.730	5.927

4. General Discussion

Brushtail possums (Trichosurus vulpecula) are widespread in Australia, as noted by early European settlers and recorded interactions with Aboriginals, from the central arid woodlands to the wet sclerophyll forests ranging the continent (Kerle, 1984). Since the first introductions in 1837, the species is now widespread in New Zealand too. Over 100 recorded introductions of possums to New Zealand occurred between 1838 to 1926 from various source populations in Australia (mainly from Victoria and Tasmania); in most cases just a small number of individuals were involved with each introduction (Pracy, 1974). When possums were introduced by the government, acclimatization societies and private owners to establish a fur trade in New Zealand, their potential as a generalist species, combined with the absence of predators and diseases, and abundance of resources, allowed them to flourish (Allendorf & Lundquist, 2003). Unfortunately this had a major negative impact on New Zealand ecosystems, as they browse native plants excessively, predate native birds and their eggs, and native invertebrates (Payton, 2000; Sadleir, 2000). From the country's economic perspective, possums affect cattle in the dairy industry as they are reservoirs for Bovine Tuberculosis (Tb) (Livingstone et al., 2015) which has been a notifiable agricultural disease since 1893 (de Lisle, 1993).

Dispersal (place of birth to reproduction) of possums is male-biased with an average distance of 5 km (P Cowan & Clout, 2000), occupying activity areas between 0.2 to 19.5 ha/ year (Glen et al., 2012). This combined with their complex introduction histories could influence their evolutionary potential in New Zealand. Additionally, reproduction between individuals introduced from different source populations may have led to novel genotypes that give possums a survival advantage to further establish and sustain populations (Sarre et al., 2014).

As brushtail possums are considered an invasive mammal species in New Zealand, my study was aimed at identifying their current population structure in New Zealand resulting from their introductions as well as extensive spread and potential admixture. This would provide a foundational insight for targeting (killing) possums more efficiently.

Unlike many studies of biological invasions around the world (Ricciardi, Steiner, Mack, & Simberloff, 2000), there was strong background knowledge available with respect to introduction histories and artificial translocations of possums in New Zealand (Montague, 2000; Pracy, 1974). However, surprisingly, there is a paucity of information regarding possum population structure and connectivity over a large scale. Considering the objective of Predator

Free 2050 (https://predatorfreenz.org/)- exterminating brushtail possums (Trichosurus vulpecula), stoats (Mustela erminea) and rats (Rattus norvegicus, Rattus rattus, Rattus exulans) from New Zealand by 2050 to protect its native, endangered species (Owens, 2017)- this is an important step to infer how past and current management efforts have altered their genetic makeup, which, in turn affects the efficiency of control measures. As far as invasive mammal pests in New Zealand are concerned, brushtail possums are high up on the priority list for eradication. For effective management of these Australian marsupials requires detailed understanding of their population structure (Taylor et al., 2004). A genetic basis (population genetics, spatial distribution of genetic variants) of this distribution would optimize management measures, while simultaneously obtaining information about resistance to control measures and barriers to connectivity depending on population responses to these measures.

Based on the potential for considerable intermixing of populations since their introduction, has there been sufficient connectivity to have a homogenize the genetic diversity of brushtail possums in New Zealand?

I aimed to answer this by incorporating genetic data generated from a single-locus mitochondrial marker and eight microsatellite loci into various population genetic analyses, indicative of general distribution of genetic diversity across the country.

4.1 Key results and implications

4.1.1 Population structure using mitochondrial haplotype variation

Locus specific PCR primers were used to amplify the D-loop / Control Region of the mitochondrial genome of brushtail possums. This is the first study to use haplotype diversity to analyse population structure of this species at a large scale. Reference whole mitochondrial genomes (separate study at Massey University) was used to design primers targeting part of the Control Region.

Mitochondrial markers are single-locus, sensitive and precise markers that produce informative genealogical data (Sunnucks, 2000). In my study, tests for DNA polymorphism showed high haplotype diversity among 209 individuals from 14 population samples in throughout New Zealand. Possums exhibit the invasive species paradox where even with successful establishment and habituation to the novel environment, they exhibit lowered levels of genetic diversity (Zalewski et al., 2011). Lowered levels of nucleotide diversity

relative to haplotype diversity suggests that only a subset of the genetic diversity was initially introduced from Australia to New Zealand.

Generally, an overall reduction in nucleotide diversity as well as haplotype diversity in all populations can be attributed bottleneck effects (Avise, 2000), which in this case, could be the local bottleneck-like effects caused by control measures (e.g.- Samples from Manaroa (n=36) with haplotype diversity, h= 0.52 and nucleotide diversity, π= 0.011). A haplotype network (Figure 2.7) illustrated the two most common haplotypes were shared among populations in most locations but these had high nucleotide base pair differences between them.
Furthermore, the haplotype network for 154 individuals for which fur colour information was available showed intermixing of lineages (Figure 2.8); in Australia fur colour is mostly correlated with spatial distribution.

My expectation that there would be little genetic structure in New Zealand was not met as results clearly indicate the presence of rare haplotypes that differentiate populations. This suggests that even with extensive artificial translocations of possums and close interactions between the various morphs, there have been barriers to their dispersal which could be ascribed to their complex colonization history.

4.1.2 Population structure using nuclear microsatellite markers

Most studies examining population demographics, genetics and biology of possums have dealt with limited spatial scale (Adams, 2013; Rouco et al., 2013; Sarre et al., 2014; Triggs & Green, 1989). It is not known whether the current genetic diversity within populations the result of initial introductions of possums from different source populations or a consequence of management efforts that were carried out over the last century. The first step is, however, to identify this distribution across the country.

Using genotypes of 275 individuals from 19 locations for seven polymorphic microsatellites, I inferred eight genetic clusters (K) with strong population structure. Some genotypes were shared by possums sampled at separate locations within regions. For example, one cluster was common in Hawke's Bay, Gisborne and Pukewharaiki; another cluster was restricted to Northland; and possums from West Coast, Tasman and Stewart Island were part of another distinct cluster. Either side of Cook Strait, there was evidence of sharing genotypes. The possum samples from Manaroa which had high mtDNA diversity also had high nuclear and fur colour diversity. Individuals from this sample were assigned to more than one genotypic cluster, suggesting the population resulted from mixing of founders from different source locations. I noted reduced diversity in my Turitea and Skyfarm samples which may have

been caused by management efforts reducing local population size. As data from these locations included some sampling over several years (2003-2017) it was apparent that there has been no recent influx of individuals that have added to the genetic diversity in these locations either by artificial dispersal or naturally- which could be due to physical barriers in the landscape features (Slatkin, 1987).

Although there is admixture of genetic variants between geographically distant locations, my study provides evidence for a well-developed population structure which goes against the hypothesis of a homogenized genetic diversity across New Zealand. This is supported by the results of pairwise F_{st} comparisons which showed that there was genetic difference between all pairs of populations except one; IBD tests indicating no overall trend of isolation by distance between the populations, as expected from their introduction history. For all populations, there was lower mean observed heterozygosity, which could be due to sampling effects (small sample size, non-uniform sampling) (Richardson et al., 2016).

The data suggest that initial introduction of possums to many locations resulted in mixing between populations, however, comparisons with natural populations revealed that the initial introduction of possums brought in only a subset of the genetic variants of brushtail possums from their source populations in Australia. However, in New Zealand it is likely that management efforts have reduced genetic diversity at some locations.

4.2 Future Research

How effective is management? What is the potential for resistance to 1080 to evolve in New Zealand? If resistance did evolve, how fast would this trait spread around the country? How do possums re-establish populations after poisoning? Would a potential vacuum effect (Efford et al., 2000) from neighbouring populations allow this or are there physical landscape barriers to gene flow?

With over 1,200 ear clip samples of possums collected from locations across New Zealand and potential to source samples from the gaps in current locations, it will be possible to enhance these data and step closer to answering these questions.

By examining historical samples from New Zealand, it should be possible to test further whether initial genetic diversity was lost during range expansion and/or subsequent management.

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