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Analysis of Mitochondrial Control Region DNA Variation in New Zealand's Brushtail Possums (*Trichosurus vulpecula*)

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Ecology at Massey University, Palmerston North, New Zealand.

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ERRATUM

Page 16, line 15 should read: around 0.8 – 1.4 kb long in vertebrates (Sbisa *et al* 1997).

Page 21, lines 20 and 21 should read: and other mammals

Page 22, line 22 should read: Although heteroplasmy has been recorded (Bermingham *et al.* 1986, Cassane *et al* 1997, Fumagalli *et al* 1996, Wilkinson and Chapman 1991) it is relatively rare (Avise *et al.* 1987).

Page 27, line 8 should read: where they occur in low proportions (Kerle *et al* 1991).

Page 43, line 8 should read: Of those six, five were observed in possums of both colours. The exception to this is haplotype 2, which was detected in grey possums only.

Page 65, line 2 should read: the control region is very A + T rich

Page 68, lines 3 and 4 should read: Gels were poured between glass plates, pre-chilled to 4°C, and run vertically.

Page 68, line 18 should read: used to confirm sequence differences by first amplifying the individual using Tv5'F and Tv5'R and then performing a sequencing reaction with the appropriate primer.

ADDED REFERENCES

Casane, D.; Dennebouy, N; de Rochambeua, H.; Mounolou, J.C. and Monnerot, M. 1997. Nonneutral evolution of tandem repeats in the mitochondrial DNA control region of Lagomorphs. *Molecular Biology and Evolution* **14**: 779-789.

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Abstract

Brushtail possums (*Trichosurus vulpecula*) were first introduced from Australia to New Zealand in 1858 to establish a fur industry. Currently numbering more than 65 million, they are recognised as the most important mammalian pest in New Zealand, because of the environmental and agricultural damage they cause. Possums act as a wildlife reservoir of bovine tuberculosis (Tb) and, as such, threaten New Zealand's multi-million dollar beef and dairy industry. Eliminating bovine Tb in livestock requires removal of contact with infected possums. This is mainly achieved through the intensive poisoning of areas of known wildlife Tb infection and the establishment around them of zones of low possum density (known as buffer zones) adjacent to at-risk farmland. Not only does this result in lower possum density, and thus fewer dispersing possums, but may also affect the movement patterns of possums.

Measurement of gene frequency differences between populations associated with a buffer zone would allow a qualitative estimate of the effect of buffer zones on limiting possum movement. The mitochondrial DNA (mtDNA) control region is an effective marker for detecting intraspecific genetic structure because it has a high mutation rate, lack of recombination and uniparental mode of inheritance.

An extensive survey of brushtail possum mtDNA control region variation in New Zealand was conducted to quantify levels of variation and thus assess the utility of the mtDNA control region as a marker for detecting genetic differentiation between possum populations. Nine haplotypes were found among 70 possums from throughout New Zealand. Most of the variation (six haplotypes) was concentrated in the North Island, and the most widespread haplotype (occurring in all four islands surveyed) was also the most common - found in 67% of possums surveyed.

The technique of single stranded conformation polymorphism (SSCP) was developed for the brushtail possum so that a quick, cost-effective and sensitive method for surveying mtDNA control region variation in large numbers of individuals was available. This assay

was applied to screen the variation in possums separated by small spatial scales associated with two buffer zones in the South Island. A total of 234 possums were screened, with 98.7% found to possess the same haplotype. The other 1.3%, all from one location, possessed a second haplotype. The extremely low levels of variation makes it highly unlikely that surveys of variation in mtDNA will be able to detect an effect of buffer zones on possum movement, at least in the South Island. Areas of higher variation, such as certain parts on the North Island, would be better candidates for testing the effect of barriers such as buffer zones on genetic differentiation between possum populations.

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Abbreviations and Symbols

S.I. (Système Internationale (d'Unités)) notation is adhered to throughout this thesis. Abbreviations used in this thesis are as follows:

A	adenine
AHB	Animal Health Board
bp, kb	base pairs, kilobase pairs
C	cysteine
CSB	conserved sequence block
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
D-loop	displacement loop
EtBr	ethidium bromide
ETAS	extended termination associated sequences
g	gravity
G	guanine
H	heterozygosity
MHC	major histocompatibility complex
MP	maximum parsimony
µl, ml, l	microlitre, millilitre, litre
mm, cm, m, km	millimetres, centimetres, metres, kilometres
mtDNA	mitochondrial DNA
M	moles per litre
ng, mg, kg	nanogram, milligram, kilogram
NJ	neighbour-joining
$	New Zealand dollars
nt	nucleotide
pM, µM, mM, M	picomolar, micromolar, millimolar, molar
PCR	polymerase chain reaction

®	registered
RFLP	restriction fragment length polymorphism
SSCP	single stranded conformation polymorphism
1080	sodium monofluoroacetate
SD	standard deviation
TAS	termination associated sequence
T	thymine
™	trademark
tRNA, rRNA	transfer RNA, ribosomal RNA
Tb	bovine tuberculosis
U	unit (of enzyme)
UV	ultraviolet light
VNTR	variable number of tandem repeats
VRA	Vector Risk Area
V	volts
W	watt

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Chapter One

General Introduction

1.1 Overview

My aim in this thesis was to determine the effectiveness of mitochondrial DNA (mtDNA) markers in studying possum movement patterns. Specifically, this involved characterising the variability among New Zealand possums in the mitochondrial control region, the development of an assay to detect this variation rapidly and cost effectively, and the application of the assay to detect the genetic effect of a potential barrier to possum movement.

The thesis is divided into four chapters: a general introduction providing background information about the motivation for the research, the application of molecular markers to ecological questions, and the characteristics of mtDNA; two main data chapters written in paper format to facilitate publication; and a final chapter synthesising the overall results and conclusions.

1.2 The possum problem

1.2.1 History of introduction to New Zealand

Brushtail possums, *Trichosurus vulpecula* (Kerr 1792), are nocturnal, arboreal, Australian marsupials of the family Phalangeridae. They are now widespread in New Zealand. During the nineteenth century they were imported into New Zealand by acclimatisation societies with the aim of establishing a fur trade to rival the then flourishing Australian fur trade. The first successful introduction of the common

brushtail possum to New Zealand was by Mr C. Basstian at Riverton in 1858 (Pracy 1962). The total number of possums imported from Australia was only 200-300 individuals, but this number was greatly bolstered by local breeding of possum stocks before liberation within New Zealand.

Protection for possums was established in 1911 due to pressure from acclimatisation societies who were keen to ensure the successful establishment of a fur trade. This protection was removed the following year due to counter pressure from orchardists, farmers and conservationists citing the damage done to crops, but was quickly reinstated in 1913 due to further pressure from acclimatisation societies. This seesawing of legislation continued until 1922 when no further importations were sanctioned, although illegal liberation continued for many years (Pracy 1962). All protection for possums was removed in 1947 due to the increasingly overwhelming evidence that possums were becoming a significant pest in the New Zealand landscape.

1.2.2 Problems caused by possums in New Zealand

1.2.2.1 Agricultural and environmental

Possums are a major pest in New Zealand (Holloway 1973), causing significant damage to crops (Spurr and Jolly 1981), pasture (Gilmore 1965) and erosion control plantings of poplars and willows (Jolly and Spurr 1981; Thomas et al. 1984). Furthermore, possums are an environmental pest, causing widespread and devastating damage to New Zealand's forests and wildlife (Cowan 1991). In a study on possum diet in the Orongorongo Valley (Brockie 1992), possums were found to browse on many native tree species, favouring kamahi, northern rata, mahoe, supplejack, fivefinger and tawa. They generally concentrate on a few favoured species, and consume them until they are either destroyed in the local region or are out of season. Only then will possums move to new, previously unfavoured species. In this way, they can strip bare a patch of forest vegetation in a few decades. Some rata/kamahi forests have been all but destroyed by possum browsing (Elder 1965; Cowan 1991). This has consequences not only for the flora of the region, but also the fauna. Possums compete with native species such as kaka (Moorhouse 1997) and kokako (Leathwick *et al.* 1983) for food; and prey on many native species, especially the eggs and young of native birds (Brown *et al.* 1993a; Sadleir 2000). Additionally, two of the poisons used to control possums, sodium

monofluoroacetate (1080) and brodifacoum, have been shown to have secondary poisoning effects on many species such as North Island brown kiwi (Robertson *et al.* 1999a; Robertson *et al.* 1999b), honey bees (Goodwin and Ten-Houten 1991) and kokako (Innes 1986). At least $40 million a year is spent on killing possums, and the vast majority of this is on poisoning with 1080. A further $12 million per annum is spent on research into reducing possum numbers (Hutching 1998).

1.2.2.2 Diseases carried by possums

Possums are further considered a pest species because they act as vectors for diseases including bovine tuberculosis, which is infectious to livestock, and leptospirosis. In the North Island, up to 80% of adult possums may be infected with leptospirosis (caused by the bacterium *Leptospira interrogans* servovar balcanica, Hathaway 1981), although the South Island appears to be free of the disease (Cowan *et al.* 2000). Possums are the primary wildlife reservoir for this disease organism in New Zealand (Hathaway 1981).

Bovine tuberculosis (Tb) is caused by the bacterium *Mycobacterium bovis*. In unmanaged cattle (its natural host species), Tb is a chronic and often fatal lung disease that is transmitted readily to other cattle. From the 1960's onwards, a concerted effort has been made to eliminate Tb from livestock in New Zealand. This has meant compulsory testing of herds and slaughter of infected animals. Initially the eradication campaign was very successful. However, by the late 1960s and early 1970s it was evident that in certain parts of the country the eradication programme was not having the desired effects (Coleman and Caley 2000). Tuberculosis was first discovered in possums in New Zealand in 1967 (Ekdahl and Money 1970), and it has since become apparent that they transmit the disease to livestock. For example, chronically infected cattle herds are often found to be associated spatially and temporally with chronically infected possum populations (Caley *et al.* 1999), while large-scale control of possums generally results in a significant reduction in the incidence of Tb in cattle in surrounding areas (Tweddle and Livingstone 1994). Tb-infected possums now occupy about 6.24 million hectares (23.6%) of New Zealand (Coleman and Caley 2000), and of the $40 million spent every year on possum control, most is spent reversing the spread of Tb in possums.

1.3 Possum biology and behaviour

For possum control to be effective, an understanding of possum biology, behaviour and population dynamics is essential. An understanding of movement patterns of possums, both short- and long-range, is important for predicting how successful a control operation will be, and how quickly recovery after control is expected. Also, dispersal of individual infected possums is usually considered the main mechanism for epidemic spread of bovine Tb (Efford 1991; Morris and Pfeiffer 1991). In addition, knowledge of the timing of the breeding season is important so that control operations can be targeted to when they will be most effective – such as when pouch young are too young to survive on their own if their mother is killed.

Currently, much effort is directed at developing new biocontrol agents to reduce possum numbers. In theory, the most effective form of biocontrol for possums in New Zealand would involve a sexually transmitted vector that is passed on by possum to possum contact (Barlow 1994). The success of such a method would depend largely on the mating behaviour and long-distance dispersal habits of possums, as artificial spread of a controlling disease to every possum population in New Zealand would not be feasible. Knowledge of the way possums disperse is integral to the manner in which biocontrol is undertaken.

1.3.1 Reproduction

Possums breed mostly between March and May (i.e. autumn), and in some parts of the country again between September and November. Females usually begin reproducing when they are one year old, although their success at this age is fairly low (8%), compared to two and three year old females (success rate = 60% and 85% respectively) (Fletcher and Selwood 2000). Gestation lasts 18 days, and possums then spend four to five months developing in the pouch, after which the young spends one to two months riding on the mother's back and continuing to suckle (How and Kerle 1995). A female possum can thus raise her young to independence within a year, and so is capable of producing an offspring every year. About 80% of females breed every year. The mean life expectancy of female possums is 6 years (although females of up to 12 years have been encountered), and thus, an average female may produce five or six offspring in her

lifetime (Fletcher and Selwood 2000). Males generally mature at two years of age, and their mean life expectancy is 6 years also (Brockie *et al* 1991; Fletcher and Selwood 2000). Possums are thus a vagile and long-lived species.

1.3.2 Home range movements

Adult possums maintain home ranges, defined by Burt (1943) as the particular area that an animal uses repeatedly for foraging, denning, mating and so forth. Home ranges vary in size depending on the habitat, and the age and sex of the possum. Estimation of home range size also depends on the way it is measured. Radiotelemetry estimates larger home ranges than live trapping (Ward 1984; Cowan and Clout 2000), and long-term studies record larger home ranges than short-term studies (Brockie 1992; Cowan and Clout 2000). This is partly because possums make seasonal movements to food sources, and partly because long-term studies have more opportunity to record the range of movements a possum will make. Nevertheless, some patterns do become apparent. Female home ranges are smaller than male home ranges in the same habitat (Green and Coleman 1984; Brockie 1992). Home ranges tend to be larger in habitats where possum density is low, or where habitat quality is poor (Cowan and Clout 2000). Home ranges are rarely exclusive, yet in areas of overlap co-dominant possums of each sex tend to avoid each other (Winter 1976). Possums generally establish a home range in the first two years of life, and once a home range has been established they are likely to occupy it for life (Cowan and Clout 2000). Movement to establish a home range differs between the sexes. Female possums are relatively sedentary, and young females usually establish home ranges next to, or overlapping with, their mother (Clout and Efford 1984). Males on the other hand are much more likely to move away and establish a home range several range lengths removed from their natal range. In a stable population this leads to a pattern where females in an area will tend to be more related to each other than to the males in the same area (Triggs 1987; Efford 1991; Ji *et al.* 2000, Cowan and Clout 2000). This is a general pattern often observed in mammals (Greenwood 1980).

Home range lengths average 295 m for males and 245 m for females (Cowan and Clout 2000) – although movements in some habitats have been recorded to be much greater. For example, in Westland, possums living in forest were found to travel up to 1200 m to

nearby pasture. Jolly (1976) recorded possums moving up to 1600 m from their native pasture habitat to a seasonal food source of apples and walnuts. Thus, 2 km is generally taken as the minimum distance a possum must move for it to be classified as dispersal, rather than extreme movements within a range (Clout and Efford 1984; Cowan *et al.* 1996).

1.3.3 Dispersal

Small proportions of individuals undertake long-distance dispersal from their natal area. Dispersal has been defined as the movement of an individual from its birthplace to the place where it reproduces (Gaines and McGenaghan 1980). Thus, by definition, dispersal movements are made by young animals although adult possums do occasionally make long distance, permanent movements (Cowan and Clout 2000; Efford *et al.* 2000). The average distance dispersed by possums in New Zealand is 5 km (Table 1.1, Cowan and Clout 2000), although movements of 10 km or more are not uncommon. Dispersal is usually achieved through a series of discrete movements over several nights (Cowan and Rhodes 1993; Cowan *et al.* 1996; Cowan *et al.* 1997).

1.3.3.1 Consequences of dispersal for possum control

Long-distance dispersal has important consequences for possum control. If possums are eradicated from an area, then initial recolonisation will depend primarily on dispersal back into the area - although the extension of home ranges by neighbouring populations (the 'vacuum effect', Efford *et al.* 2000) will also have an effect. Long-distance dispersal also has important consequences for the spread of diseases such as bovine Tb. Bovine Tb in New Zealand is currently restricted to 5 major areas: the central North Island, Wairarapa, Westland, north Canterbury, and Otago; and in at least a further 15 discrete areas throughout the North and South Islands. Together, these areas are called Vector Risk Areas (VRAs, Figure 1.1). They comprise about 23.6% of New Zealand and contain 75% of New Zealand's infected cattle herds and reactor cattle (Coleman and Livingstone 2000). The Animal Health Board undertakes to contain and eradicate Tb from these areas. However the dispersal of infected possums into areas previously free from the disease can seriously hinder this containment goal. Therefore, a good understanding of the dispersal capabilities and tendencies of possums will have a significant impact on the way control is implemented.

Table 1.1 Published estimates of dispersal by *Trichosurus vulpecula* in New Zealand as found by several direct studies. N/D = not determined, [1] – a further 60% of possums were of unknown age, [2] – only juvenile possums were monitored, [3] – most immigrants were nominally adult (≥ 20 months), [4] – most immigrants were 1 – 2 years old.

HABITAT TYPE	MONITORING METHOD	PROPORTION OF INDIVIDUALS THAT DISPERSED (%)	AVERAGE DISPERSAL DISTANCE (km)	LONGEST DISPERSAL EVENT (km)	PROPORTION OF DISPERSERS MALE (%)	PROPORTION OF DISPERSERS JUVENILE (%)	REFERENCE
Beech forest	Live-trapping	N/D	6.0 ± 3.5	10.0	100	100	Clout and Efford 1984
Podocarp-Broadleaf forest	Live-trapping	N/D	3.9 ± 2.0	9.0	90	10 [1]	Clout and Efford 1984
Mixed: native forest, scrub and pasture	Radio-telemetry n=97	20	3.7	11.6	73.7	100	Cowan and Rhodes 1993
Mixed: poplars, pasture and forest	Radio-telemetry n=38	10.5	3.8 ± 1.4	5.0	100	N/D	Thomas *et al.* 1984
Podocarp-rata-broadleaf forest	Radio-telemetry n=29	22.2	4.1 ± 2.3	6.8	83.3	N/D [2]	Ward 1985
Farmland	Radio-telemetry n=61	19.6	5.5 ± 2.9	11.5	75	N/D [2]	Cowan *et al.* 1996
Farmland	Radio-telemetry n=60	25	5.3 ± 3.2	12.8	86.7	N/D [2]	Cowan *et al.* 1997
Podocarp-Broadleaf forest	Live-trapping	N/D	N/D	N/D	74	N/D [3]	Efford 1998
Mixed: orchard and native forest	Extinction trapping	N/D	N/D	N/D	53.4	N/D [4]	Little and Cowan 1992

Figure 1.1 Map of New Zealand showing Tb vector risk areas (shaded) and vector-free areas (unshaded), as at November 1998 (after Coleman and Livingstone 2000).

1.3.3.2 Dispersal is sex-biased

A great deal of research has been undertaken to understand possum dispersal, although the results have not been completely consistent. Males undertake most long-distance dispersal events. This sex bias in dispersal has been reported in many studies (Table 1.1) and for mammals in general (Greenwood 1980; 1983). In two related studies, Cowan *et al.* (1996; 1997) radio-tagged juvenile possums on farmland in Hawkes Bay. In the first, radio-transmitters were attached to a total of 61 (34 male and 27 female) newly independent juvenile possums from three habitats: swamp, willows and farmland. They found that 12 (20%) of the tagged possums moved more than 2km from their original site of capture, and this was regarded as dispersal (Cowan *et al.* 1996). Males dispersed more often than females (26% vs. 12%) although this difference was not statistically significant. Habitat also had an effect, with most dispersers being those that originated from farmland. The dispersal distances recorded ranged from 3 to 11.5 km, and this was usually achieved through a series of distinct movement episodes. In the second study (Cowan *et al.* 1997), the dispersal of juvenile possums was studied at the same site, after a control operation had reduced the possum population on the study site by approximately 90%. A total of 60 (39 male and 21 female) juvenile possums were radio-tracked. In this case, 25% of possums were found to undertake movements of 2 km or greater from their original site of capture. Males moved more than females (33% vs. 10%), but again the difference was not statistically significant. Possums from farmland habitat again dispersed more than possums from the other two habitats, but the difference was not significant. Dispersal distances ranged from 2 to 12.8 km, which was again often achieved by several discrete movements. Taken together, these two studies showed similar patterns of dispersal, suggesting that control, and hence density, does not affect dispersal tendency in possums. In a separate 15-year live-trapping study in the Orongorongo Valley, most resident animals were tagged, thus most untagged individuals were immigrant possums. Seventy four percent of the immigrants were males (Efford 1998). Clout and Efford (1984) found an even more extreme level of male-biased dispersal, where 12 out of 13 (92%) of dispersing possums were male. However, not all studies have found a sex-bias in dispersal rates. Natural immigration onto a small 5-hectare island in Kerikeri, connected to the mainland by a 200 m long causeway, was recorded for eleven years (Little and Cowan 1992). In this study, males and females invaded the area equally, in apparent contrast to most studies of both dispersal and home range movements. It was suggested that the possums were attracted

to the island by fruit growing in an orchard, and because such short distances were involved, females were just as likely to undertake the trip.

Despite the previous example, a bias towards greater dispersal by males is generally accepted. This has a number of ramifications for both traditional control and newer biocontrol methods. Firstly, if recolonisation after a control operation is facilitated primarily by dispersal, then one could expect that a recovering population would initially have a male bias. This has indeed been found in studies of recovering populations (Clout and Efford 1984). Secondly, as with home range movements, female philopatry will result in higher levels of relatedness amongst females than males. Nonetheless, although females disperse less often, when they do move, they travel disproportionately long distances – the two longest recorded dispersal events (31 and 41 km) were both made by females (G. Pannet pers. comm. to M. Efford, reported in Efford 1991). Females are also much more likely then males (50% vs. 12.5%) to make multiple dispersal movements, over several nights, before settling in an area (Efford 1991). Females may therefore be very important in the spread of diseases such as Tb.

1.3.3.3 Dispersal is age-biased

Most of the above studies, whether or not they found sex-biased dispersal, agreed that it is juvenile possums that undertake long distance dispersal (Table 1.1, Clout and Efford 1984; Little and Cowan 1992; Cowan *et al.* 1996; Cowan *et al.* 1997). Dispersal in possums seems to be triggered by sexual maturation (usually between the ages of 9 and 14 months), as it is during this process that most possums undergo long-range movements (Cowan and Clout 2000). A possum population in the Orongorongo Valley was encountered where some immigrant possums (both males and females) were about 20 months in age (Efford 1998). It appears that most possums in the Orongorongo Valley do not mature until they are 20 – 24 months of age, suggesting that it is the onset of sexual maturation, rather than purely age, that triggers dispersal.

1.3.3.4 Methods used to study dispersal

All of the studies described above have relied on one of two methods to investigate dispersal: mark and recapture or radio-telemetry. While useful, they have a series of well-recognised limitations. They are labour intensive, and therefore expensive and time consuming. More importantly, they may provide biased estimates. Firstly, males are more susceptible to initial live capture than females, but females are more

susceptible to recapture than males. Analysis of preliminary live-capture records from a study by Coleman and Green (1984) suggested a male bias in the population. However subsequent extinction trapping revealed the absolute sex ratio to be close to 1:1 (Coleman and Green 1984). The proposed reason for this greater susceptibility to initial capture in males is that males are the more mobile sex, and so are more likely to encounter traps. This bias will mean that males are sampled more frequently than females in studies of possum dispersal. Secondly, for mark-recapture studies, long-distance dispersal is difficult to detect if the dispersal event carries the individual out of the study area (Koenig *et al* 1996). Under such circumstances, recorded dispersal distance distributions can become biased towards shorter movements. Local mark-recapture studies really provide estimates of survival rates, which are affected by both emigration and mortality. Radiotracking individuals goes some way to eliminating this bias (Mossman and Waser 1999), but often only small numbers of individuals can be monitored. Radiotelemetry estimates can still be somewhat biased towards shorter movements, as it becomes increasingly difficult to radio-locate individuals with increasing distance from the central study area. If females really do disperse further (but less frequently) than males, then female long-distance dispersers would be less likely to be recovered/recorded than males staying closer to the core study area. This would exaggerate the reported sex bias of dispersal (Efford 1991). Thirdly, long-distance dispersal is a relatively rare event, and both mark-recapture and radio-telemetry are biased towards recording common events. Very rare events, such as extremely long distance dispersal, could easily be missed altogether unless tracking is done very frequently, yet these events would still have important impacts on population dynamics. Fourthly, the recorded distributions of age at onset of dispersal may be biased by the fact that several studies selectively radio-tagged juvenile animals, and so may not reflect the dispersal tendencies of the population as a whole (Efford 1991).

Another way to study dispersal is to apply a molecular marker to quantify gene frequency differences within and among populations (Slatkin 1985; 1987; Neigel *et al* 1991; Neigel 1997), and thus gain an indirect estimate of gene flow. If populations are genetically similar, this suggests high levels of dispersal and gene flow, whereas significant population subdivision is considered as evidence for limited dispersal. For this type of measure to be of use in the study of possum dispersal, it is first necessary to quantify levels of genetic variation within and between possum populations to ensure

that sufficient variation exists. It will be of further benefit to determine whether a given molecular marker will be sensitive enough to detect gene frequency differences between populations separated by geographical or physical barriers to dispersal. Previous genetic analyses of brushtail possum populations suggest that there are reasonably high levels of genetic variation within and between populations (Taylor and Cooper 1998; Lam *et al* 2000; Aitken *et al* in press) although no study to date has specifically assessed the potential for a molecular marker to measure dispersal distances in brushtail possum.

1.4 Genetic analyses of *Trichosurus vulpecula*

Several studies of the population genetics of brushtail possums have been conducted since 1989. Levels of variation have varied widely, partly due to the type of molecular marker used. Consequently, potential for the development of effective genetic markers for population level analyses will be highly dependant on the nature of the marker chosen - more variable markers have the potential for more accurate detection of movement between populations.

1.4.1 Allozymes

The first large-scale population genetics study of the brushtail possum was conducted using 45 allozyme loci, of which nine (20%) were polymorphic in the Australian and New Zealand populations studied (Triggs 1987). Allozymes revealed low levels of divergence between Australian populations in New South Wales, Victoria, Tasmania and South Australia (Nei's D[1] = 0.004-0.017) and a mean observed heterozygosity, averaged over all populations, of 0.044 (Triggs 1987; Triggs 1990). When South Australian possums were omitted, leaving only those populations thought to have been sources of New Zealand possums, heterozygosity was reduced to 0.039, which was similar to the average heterozygosity of New Zealand populations (H = 0.041), suggesting New Zealand possums were most likely derived from these populations (Triggs and Green 1989). No alleles were found in New Zealand that did not occur in Australia. In Australia, Tasmanian possums had lower heterozygosity (H = 0.029) than

[1] Nei's (1978) unbiased genetic distance (D).

mainland possums (H = 0.044), which correlated with New Zealand populations that had greater than 50% black possums (H = 0.033) and those that had less than 50% black possums (H = 0.049). This suggested that the origins of New Zealand populations are still reflected in allele frequencies, and can be roughly estimated by coat colour (Triggs 1990, Triggs and Green 1989). Allozymes lack the resolution to completely resolve the degree of genetic variation and subdivision amongst New Zealand possum populations (Taylor *et al.* 1998), because of the low overall levels of allozyme variation and divergence among both Australian and New Zealand populations.

1.4.2 Microsatellite DNA

Microsatellite markers were developed for *Trichosurus vulpecula* in 1998 (Taylor and Cooper 1998). Of the eleven loci successfully amplified in this study, eight were polymorphic in New Zealand populations. High levels of diversity were also found among Australian possums. The polymorphic loci had large numbers of alleles per locus (4-15) and high heterozygosity (H_E = 0.11-0.83), suggesting that these loci would be very useful for population genetic studies in New Zealand and Australia. Six of these loci were recently used in a genetic analysis of the mating system of *Trichosurus vulpecula* (Taylor *et al.* 2000). Again, a high level of genetic diversity was found, with an average of 11.8 alleles per locus, and heterozygosity of 0.83.

A survey of variation over a small spatial scale among seven populations in the Tararua Ranges (separated by a total of 24 km) using four variable loci is currently in progress (Q. Hudson, S. Sarre and P. Cowan, unpublished data). Levels of variation are high, with between five and 23 alleles observed at each locus. This study has found little evidence of structuring amongst males, even over distances of 24 km, and only low levels of structuring amongst females over similar distances. The four loci, and a further four loci, are currently being used to survey the genetic variation occurring across a buffer zone in Canterbury (discussed further in section 4.4). This work is not completed, but levels of variation appear to be high enough to make inferences about genetic structure between sites separated by 60 km or less.

A MHC-linked microsatellite marker (a CA-repeat) has also been developed for brushtail possums (Lam *et al.* 2000). This locus has a moderately high level of

variation, with six (H_E = 0.729) and nine (H_E = 0.770) alleles observed at Hohotaka and the Orongorongo Valley respectively. A total of 11 alleles were found amongst the two populations. The MHC is an important genomic region because it is involved in the immune response. The MHC-linked marker described by Lam *et al.* (2000) has a high level of heterozygosity for both populations analysed, suggesting it will be a potentially useful marker for population level studies, including the study of relationships between MHC haplotypes and Tb susceptibility.

The main drawback of using microsatellite markers for population genetic studies is that their mode of evolution is not widely understood (Jarne and Lagoda 1996). Several conceptual models of the mutation process have been developed and applied to microsatellite data (Kimura and Crow 1964; Ohta and Kimura 1973; Di Rienzo *et al.* 1994; Garza *et al.* 1995), but it is far from clear which, if any, correctly predict the nature of the mutational process in microsatellite alleles. An accurate model of the mutation process is important, as it allows one to assess the relative importance of different types of change, establish how closely alleles are related, and predict the likelihood and extent of homoplasy (Lunt *et al.* 1998). These same limitations apply to minisatellite markers, discussed below.

1.4.3 Minisatellite DNA

Minisatellite DNA fingerprinting has been used to analyse the behaviour and mating system of brushtail possums, using three minisatellite probes (Sarre *et al.* 2000; Ji *et al.* 2001). Paternity of pouch young was determined by assigning maternal and paternal bands in the minisatellite profile, and rejecting all potential sires as the father if they did not contain every paternal band present in the young. Of 80 pouch young examined, paternity could be unambiguously assigned to a specific male in 54 (67.5%) cases (Sarre *et al.* 2000). Paternity was not distributed evenly among males, instead only 53.5% of all adult males sired young, and these males, on average, mated with more than one female per year, suggesting a polygynous mating system. Average relatedness within populations was determined by levels of bandsharing - bandsharing is higher in related animals because they will share alleles by descent. Bandsharing was higher among females than among males, confirming that males are the dispersing sex (Ji *et al.* 2001). Average bandsharing within possum populations was fairly high (0.41-0.71),

suggesting that founder effects are still evident in New Zealand possum populations for these multilocus nuclear DNA markers (Ji *et al.* 2001). Minisatellite profiling may be useful for population genetic surveys, further work with these markers is warranted.

1.4.4 Mitochondrial DNA

1.4.4.1 Cytochrome b

A limited analysis of sequence variation in the cytochrome *b* gene in possums detected only one base pair change between two individuals (Aitken 1997). A more extensive RFLP survey of 13 individuals revealed no variation between possums. As such, cytochrome *b* is not likely to be useful as a marker for population genetic studies, but may be more useful for studies of interspecific variation (Aitken 1997).

1.4.4.2 Control region - VNTRs

Sequencing of the entire mtDNA control region of possums identified three repeat sequences (VNTRs - variable number of tandem repeats) in the right variable domain, responsible for length variation and heteroplasmy in the control region (Aitken 1997; Aitken *et al* in prep). The length of each of the repeat arrays was quite different in the two individuals analysed: 7-9 for repeat one (a di-nucleotide repeat), 7-14 for repeat two (a deca-nucleotide) and 18-23 for repeat three (an octo-nucleotide). Analysis of repeat three in possums from the Tararua Ranges, Banks Peninsula and Stewart Island indicated that populations from different geographic regions contained different ranges of repeat array length, suggesting that this may be a useful marker for population analyses (Aitken *et al.* in prep), so long as repeat length is stably inherited from mother to offspring. Several limitations to using mtVNTRs in population analyses have been identified, most importantly that the mechanisms generating mtVNTR diversity are largely unknown (as for microsatellite and minisatellite markers, discussed in section 1.4.2), and that the mtVNTR size classes assayed by PCR may not accurately represent the population of mtVNTRs present within the organism (Lunt *et al.* 1998). Thus, although mtVNTR markers may contain the variability appropriate for use in some population level analyses, their use should be approached with caution.

1.4.4.3 Control region - non-repetitive regions

One molecular marker that has not been extensively surveyed for population-level variation in brushtail possums is the non-repetitive segment of the mitochondrial control

region, yet this region contains high levels of variation in other animals (Saccone *et* al 1991, Norman *et* al 1994, Gemmel *et al* 1996, Meyer *et al* 1999). It has been shown to have sufficient sensitivity to detect the genetic effects of such parameters as geographic and physical barriers to movement, and sex-biased dispersal (reviewed in section 1.6). It also has many of the characteristics of an ideal molecular marker, as discussed below (section 1.5.3).

1.5 Mitochondrial DNA

1.5.1 General characteristics

The mitochondrial genome of most vertebrates is a closed circular loop of DNA 16-20 kilobase pairs (kb) in size (Gemmell *et al.* 1996). It exhibits extreme conservation of gene content, with mtDNA molecules from insects, echinoderms and vertebrates having two ribosomal RNA genes, 22 transfer RNA genes, and 13 genes involved in oxidative phosphorylation within the mitochondrion (Figure 1.2, Harrison 1989; Janke *et al.* 1994; Taanman 1999). The mtDNA molecule also contains a control region, roughly 0.8 kb long, with non-coding sequences that are involved in initiation of transcription and the initiation and termination of replication. This non-coding region is the most rapidly evolving part of the mtDNA (Upholt and Dawid 1977), and contains both conserved regions as well as regions of high variability (Janke *et al.* 1994).

Animal mtDNA has many important features that make it amenable to studies of gene flow and dispersal. First, it is non-recombining: mtDNA is inherited solely down the female line and so is haploid. Mutations occurring in different individuals are not recombined during sexual reproduction, and so lineages can be traced phylogenetically (Moritz *et al.* 1987). The entire mtDNA genome represents a single, completely linked, genetic unit with multiple alleles (Taberlet 1996). Stochastic processes (i.e. mutation and genetic drift) will have an especially important role in determining frequencies of mtDNA haplotypes, as new variants will not be created or lost due to recombination (Harrison 1989). However, a mtDNA phylogeny reflects only one of many possible

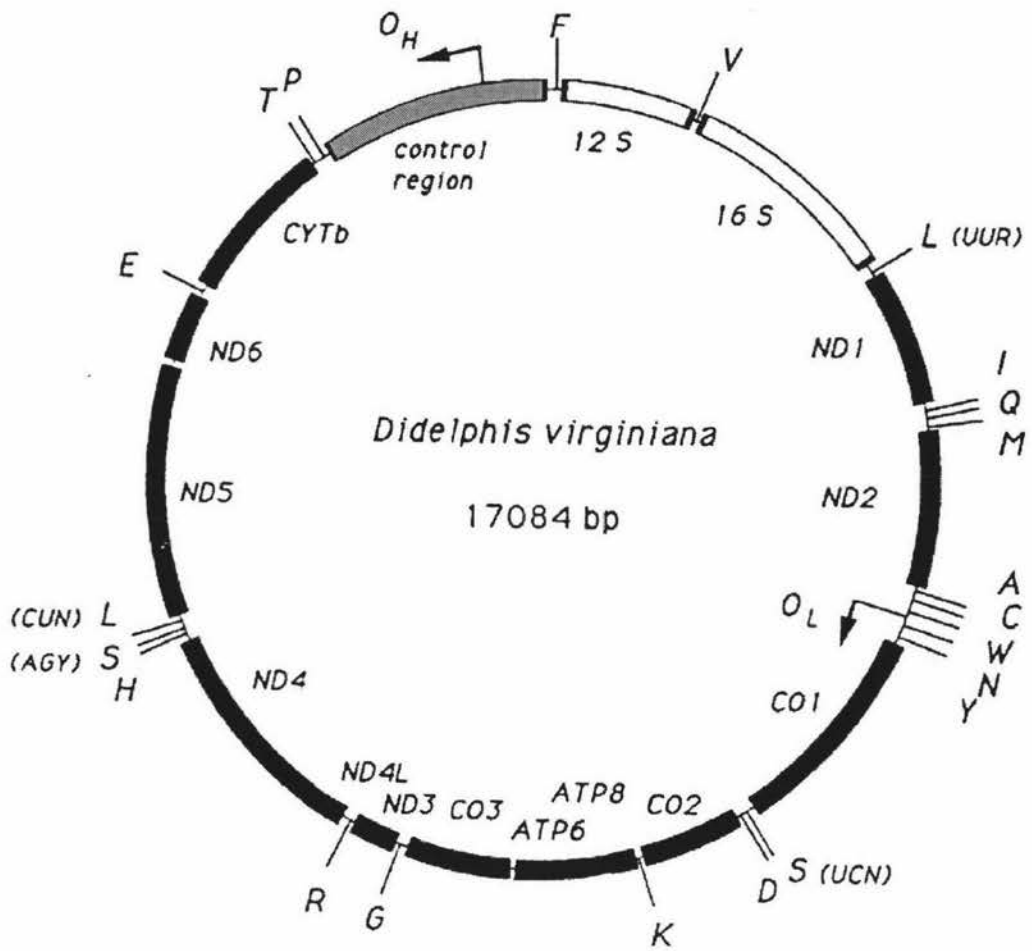


Figure 1.2 Map of the mitochondrial genome of the American opossum *Didelphis virginiana* (after Janke *et al.* 1994). The control region is shown in grey, and each tRNA is identified by its one-letter amino acid code.

genetic pathways, and as such will not be as informative as a phylogeny constructed from multiple nuclear genes (Hartl and Clark 1997). For example, after twenty generations, the fraction of all possible genetic pathways represented by the female to female transmission route is less than one-millionth of that collectively available to an autosomal gene (Avise 1995). Second, the mtDNA genome is present as multiple copies in every cell, so older or less well preserved samples will usually still yield good quantities of mtDNA compared with single copy nuclear DNA, particularly since the

mtDNA genome is relatively small in most animals. Mitochondrial DNA is ubiquitously distributed such that homologous comparisons can be made for a wide range of species (Avise *et al.* 1987). Third, mtDNA has a simple genetic structure. It is free from introns, psuedogenes, and transposable elements and, with the exception of the control region, free of repetitive DNA; all of which can complicate studies using nuclear markers (Avise *et al.* 1987). Both the tRNA and rRNA molecules are uncharacteristically small, and some protein genes overlap (Taanman 1999). Fourth, mtDNA evolves rapidly, especially the non-coding control region. Evolution at the sequence level is thought to be 1-10 times faster than for single-copy nuclear DNA (Brown *et al.* 1979), although this varies between species (Moritz *et al.* 1987). The control region evolves especially rapidly, with some sections evolving, on average, about 4 – 5 times faster than the entire mtDNA molecule (Brown *et al.* 1993b). This rapid rate of evolution promotes extensive intraspecific polymorphism that can be used to distinguish recently separated populations (Avise *et al.* 1987). Lastly, because of its maternal mode of inheritance, mtDNA has as little as one quarter the effective population size of nuclear genes. This means that stochastic lineage sorting will result in random extinction of many mtDNA haplotypes (Avise *et al.* 1984), but also means that it is possible to trace the phylogenetic history of matrilines back through time, and answer the question "how far back in time might pairs of extant organisms have last shared a common female parent?" (Avise *et al.* 1984). This is achievable because, under normal demographic conditions, coalescent processes ensure phylogenetic connections among genotypes via vertical pathways of descent (Avise 2000). Looking into the past, mtDNA haplotypes eventually coalesce to common ancestors. Stochastic lineage sorting can be very rapid, especially when population sizes (of reproductive females) are small. In rapidly expanding populations however, lineage extinction is rapidly slowed (Avise *et al.* 1984; Boileau *et al.* 1992).

1.5.2 The mitochondrial control region

The mtDNA control region, as discussed in section 1.5.1, is the region that contains sequences that are involved in initiation of transcription (heavy-strand [HSP] and light-strand promoters [LSP]) and replication (O_H and O_L) (Clayton 1982; Clayton 1991). It is located between the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$. In animal mtDNA the non-coding control region contains the displacement-loop (D-loop), which is a three-

stranded structure containing the heavy (H) strand, the light (L) strand and a short nascent H strand that has its 5' end located at the origin of H-strand replication (O_H) (Clayton 1991). It is from this point that replication of the heavy strand proceeds.

Three sections of the control region have been recognised: the left ETAS domain, which contains one or two extended termination associated sequences (ETAS, Sbisa *et al.* 1997); the central conserved domain; and the right CSB domain, that contains the site for initiation of heavy-strand synthesis (O_H), and two or three conserved sequence blocks (CSB's, Figure 1.3, Saccone *et al.* 1991; Taberlet 1996). The central conserved domain has extensive sequence similarities between species, and appears to evolve at roughly the same rate as the mtDNA protein coding genes (Brown *et al.* 1986; Gemmell *et al.* 1996). Beyond this central domain there is little sequence conservation between species, although it has been suggested that secondary structure is relatively conserved throughout the mitochondrial genome (Brown *et al.* 1986; Hoelzel *et al.* 1991). Brown *et al.* (1986) and Saccone *et al.* (1985) have shown that sequences in the right CSB domain of various species are capable of forming cloverleaf secondary structures. Similar secondary structure from various species can also be identified in the ETAS domain, associated with the sites for termination of D-loop synthesis (TAS, Brown *et al.* 1986; Sbisa *et al.*1997). Thus, the left ETAS and right CSB domains appear to be under structural and functional constraint, if not sequence constraint.

1.5.3 Characteristics of an ideal molecular marker

To be useful in population analyses, a molecular marker ideally possesses many of the following characteristics. It needs to have a mode of inheritance and evolution that is well understood, such that the variation it detects is easy to interpret and replicate. It needs to evolve rapidly enough, and contain sufficient variation, to show variation over small time scales or over small geographic distances, but not so much that intraspecific comparisons cannot be made (Bossart and Pashley Prowell 1998). To facilitate intraspecific analysis, the marker should not be under the influence of selection or recombination. A small effective population size will increase the resolving power of the marker, which is useful for the study of large, widespread or rapidly expanding populations. Lastly, the marker should be easily and inexpensively isolated and characterised so that large studies involving many individuals can be performed. The

control region of mitochondrial DNA (mtDNA) possesses most of these characteristics, and has several further advantages that make it particularly useful for analysis of population structure.

1.6 Genetic analyses of population structure using mtDNA

Genetic methods using mtDNA, and especially the highly variable control region, have been used widely to study gene flow and dispersal, and to quantitate the effects of barriers to animal movement. They have been found to be cost effective and rapid, and to provide potentially non-biased estimates of gene frequency differences between populations.

1.6.1 Sex-biased dispersal

Mitochondrial markers can easily detect sex-biased dispersal because mtDNA is maternally inherited. Females thus have great potential to homogenise mitochondrial populations through dispersal because a migrating female can leave copies of her mtDNA haplotype in different populations as she, and her offspring, migrates. In comparison, the mtDNA haplotype carried by a male is available for sampling during his lifetime only, and only in the location he is present in when sampled. So if, despite this fact, females exhibit greater mitochondrial genetic structure between populations than males, it would be reasonable to assume that dispersal is mediated by males (O'Corry-Crowe *et al.* 1997; Escorza-Trevino and Dizon 2000).

Escorza-Treviño and Dizon (2000) recently used 379 base pairs (bp) of mtDNA control-region sequence from 113 individuals from 11 geographic regions, to estimate, among other things, sex-biased dispersal of Dall's porpoise. When pairwise F_{ST} comparisons, stratified by gender, were made between populations from different regions, they showed greater differentiation among females from different regions than among males. This implied that there were different dispersal rates between the sexes, and that males dispersed more often than females. The study also found lower levels of genetic partitioning with microsatellite markers (biparentally inherited) than with mtDNA (maternally inherited), which, in conjunction with the mtDNA patterns, supports male-biased dispersal (Escorza-Trevino and Dizon 2000). This pattern of male-mediated gene flow causing genetic structure with mtDNA makers but not nuclear markers has

also been found in many other studies (Melnick and Hoelzer 1992; FitzSimmons *et al* 1997; Ishibashi *et al* 1997; Firestone *et al* 1999; Nyakaana and Arctander 1999). Conversely, a study of gene flow in red grouse found a lack of population differentiation in the mitochondrial control region, but hypervariable microsatellite markers detected localised population subdivision among the same populations (Piertney *et al* 2000). This was explained by the fact that grouse cocks show high levels natal philopatry while females exhibit high levels of dispersal, which prevents mtDNA divergence (Piertney *et al* 2000), although one would expect that female-mediated gene flow would tend to homogenise both autosomal and mitochondrial variation simultaneously.

1.6.2 Effects of geographic distance on population structure

Chenoweth *et al.* (1998) used mtDNA control region sequences to infer a relationship between gene flow and geographical distance in the Australian barramundi (*Lates calcarifer*). Of the 63 haplotypes they identified, many were shared within regions, whereas there was no sharing of haplotypes between regions. This was in general agreement with an earlier allozyme-based study, although mtDNA was able to detect structure at a finer scale. Other species in which a correlation between mtDNA control region genetic relatedness and geographic distance have been found include fish (Stepien 1999; Riginos and Nachman 2001), invertebrates (Ross 1999), birds (Pitra *et al* 2000; Wennerberg 2001), marsupials (Houlden *et* al 1999; Pope *et al* 2000) and mammals (Pichler *et al* 1998; Waits *et al* 1998).

1.6.3 Effects of barriers on population structure

In a recent study of mtDNA control region sequences in the black surfperch, *Embiotoca jacksoni*, a temperate reef fish lacking a pelagic larval stage, Bernardi (2000) found a major phylogenetic break at Santa Monica Bay - a sandy expanse that appears to inhibit adult and larval dispersal. Phylogenetic divisions were also found between localities separated by deep-water channels, also known as barriers to dispersal in this species. Santa Monica Bay had not previously been recognised as a potential barrier to dispersal, showing that genetic methods can accurately identify ecological or historical barriers to movement. The mtDNA control region is a sensitive marker for detecting the effect of various potential barriers to gene flow, such as water expanses (Edwards 1993; Eizirik

et al 2001), land formations (Jones and Quattro 1999; Roman *et al* 1999; Lehmann *et al* 2000), habitat fragmentation (Fowler *et* al 2000; Andayani *et* al 2001) and physical characteristics of the environment such as temperature and salinity (Riginos and Nachman 2001). In fact, Avise and Walker (1999) have shown that 56% of 252 vertebrate species surveyed with mtDNA markers could be separated into two matrilineal phylogroups that mostly (96%) correlated also with geographic partitioning.

1.6.4 Utilising mtDNA control region markers to assess possum population genetics

The application of molecular methods to quantify levels of variation in New Zealand possum populations, and to assess whether these markers are sensitive enough to detect potential barriers to dispersal, will allow an assessment of whether this marker can be used to quantify gene flow and hence infer movement.

Several studies have compared the sensitivity of mtDNA markers with allozymes or microsatellites, for the detection of gene frequency differences between populations. Mitochondrial DNA was generally found to be more sensitive than allozymes. Use of microsatellite studies in association with mtDNA is often a good strategy for the study of dispersal, as this allows discrimination of sex-biased patterns of movement: mtDNA will detect female-mediated movement, and microsatellites (and other nuclear markers) will detect total movement (reviewed in section 1.6.1).

MtDNA studies of the control region can be complicated by heteroplasmy, where nonidentical mtDNA molecules coexist in the same individual (Lunt *et al.* 1998). Although heteroplasmy has been recorded (Bermingham *et al.* 1986), it is relatively rare (Avise *et al.* 1987). There is usually a close approximation to mtDNA homoplasy in somatic cells in most individuals because, although mtDNA mutations are common, they are lost or reach fixation within a few generations (Avise 2000). Size heteroplasmy has been found in brushtail possums (Aitken 1997), and it appears to occur exclusively in the repeats at the 3' end of the control region. This region has thus been avoided in the present study of mtDNA variation in possums.

1.7 Project description and justification

Genetic methods analysing mtDNA control region sequence variation within and among populations offer a new and complementary way in which to study the population genetics and movement patterns of brushtail possums. Several studies have addressed the development of population genetic markers for possums (discussed in section 1.4). The mtDNA control region was chosen as a potential marker as a two-pronged approach to developing population markers for management.

The hypervariable portions of the control region offer the most promise for the development of population genetic markers for several reasons. Firstly, a recent genetic examination of genetic variability of mtDNA in brushtail possums found no variation in the cytochrome *b* gene (Aitken 1997). Although there was variation in the repeat regions (VNTRs) these were difficult to interpret because of potential heteroplasmy (reviewed in section 1.4.4.2, Aitken 1997). Secondly, the conserved sequence blocks in the 3' end of the mtDNA control region show a high degree of genetic conservation between species, and as such are unlikely to provide sufficient resolution of recent evolutionary events.

The goals of this research were to:

- Characterise the hypervariable portions of the control region in possums.
- Assess overall levels of mtDNA control region variation in New Zealand possums.
- Develop a rapid and inexpensive method to quantify this variation.
- Assess the effect of a potential barrier to possum movement on mtDNA variation in possums.
- Use the results of mtDNA surveys to make inferences about movement patterns in possums in New Zealand, over both large and small geographic scales.

Chapter two describes the levels of mtDNA genetic variation found in possum populations sampled from throughout New Zealand, and how this was determined by direct sequencing of 900 bp of the mtDNA control region. The fit of Australian

samples into a phylogeny of New Zealand possums is described, and the relationships between genetic variability, location of sample site, and coat colour are discussed.

The third chapter reports on the development of a rapid, sensitive, and cost effective method to quantify genetic variation in possums – namely using single strand conformation polymorphisms (SSCPs) to detect sequence differences. This technique was used to examine population substructure and movement of possums across poison buffer zones.

Chapter four summarises the current research and results obtained, evaluates the success of the approach chosen for detecting gene frequency differences in possum populations, and suggests future directions for this work.

Chapter Two

Population Genetic Survey of Possums in New Zealand

2.1 Introduction

2.1.1 Taxonomy of possums in Australia

Brushtail possums (*Trichosurus vulpecula* Kerr 1792) were first successfully introduced to New Zealand in 1858 in order to establish a fur trade (Pracy 1974). These possums were sourced from Australia, where they occur in five disjunct regions: northern Australia, eastern Australia, central Australia, Tasmania, and south-western Australia (Figure 2.1, How and Kerle 1995). The systematics of possums in Australia is a contentious issue, with some authorities classifying *Trichosurus vulpecula* into as many as eight subspecies (Figure 2.1, Ogilby 1835; Troughton 1941; How and Kerle 1995). The division of brushtail possums into subspecies has been based largely on geographic distribution and morphological characters, most notably coat colour, furriness of the tail, and size. For example, possums from Tasmania (often known as *T. vulpecula fuliginosus*) vary from grey to black, while possums from Queensland (classified as *T. v. johnstonii*) can be reddish brown in colour. In South Australia (where they are classified as *T. v. vulpecula*) and Tasmania, possums can weigh up to 4.5 kg, but in northern Australia (classified as *T. v. arnhemensis*) they are one third this size. Despite these differences, recent genetic investigations by Kerle (1983), Kerle *et al.* (1991) and Triggs (1990) have found only limited support for subspecific classifications of *Trichosurus vulpecula*. In an extensive analysis of the systematics of the brushtail possum, using morphological characteristics, karyotypes, allozyme and ecological data, Kerle *et al.* (1991) recommended the retention of northern populations as a subspecies,

T. v. arnhemensis, on the basis of morphological and behavioural characteristics. They also suggested the retention of Tasmanian and south-western Australian populations as subspecies based on geographic isolation, although these populations were not genetically distinct. They found no support for further subdivision of *T. vulpecula*, attributing morphological variation to phenotypic plasticity occurring in response to prevailing environmental conditions, and to clinal variation. Triggs (1990), in an allozyme study, found that Tasmanian brushtail possums were no more genetically differentiated from mainland populations than mainland populations were from each other. She considered that differences in size, coat colour and heterozygosity were part of a cline throughout Australia, conforming to Bergman's Rule - races from warm regions are smaller than races from cold regions.

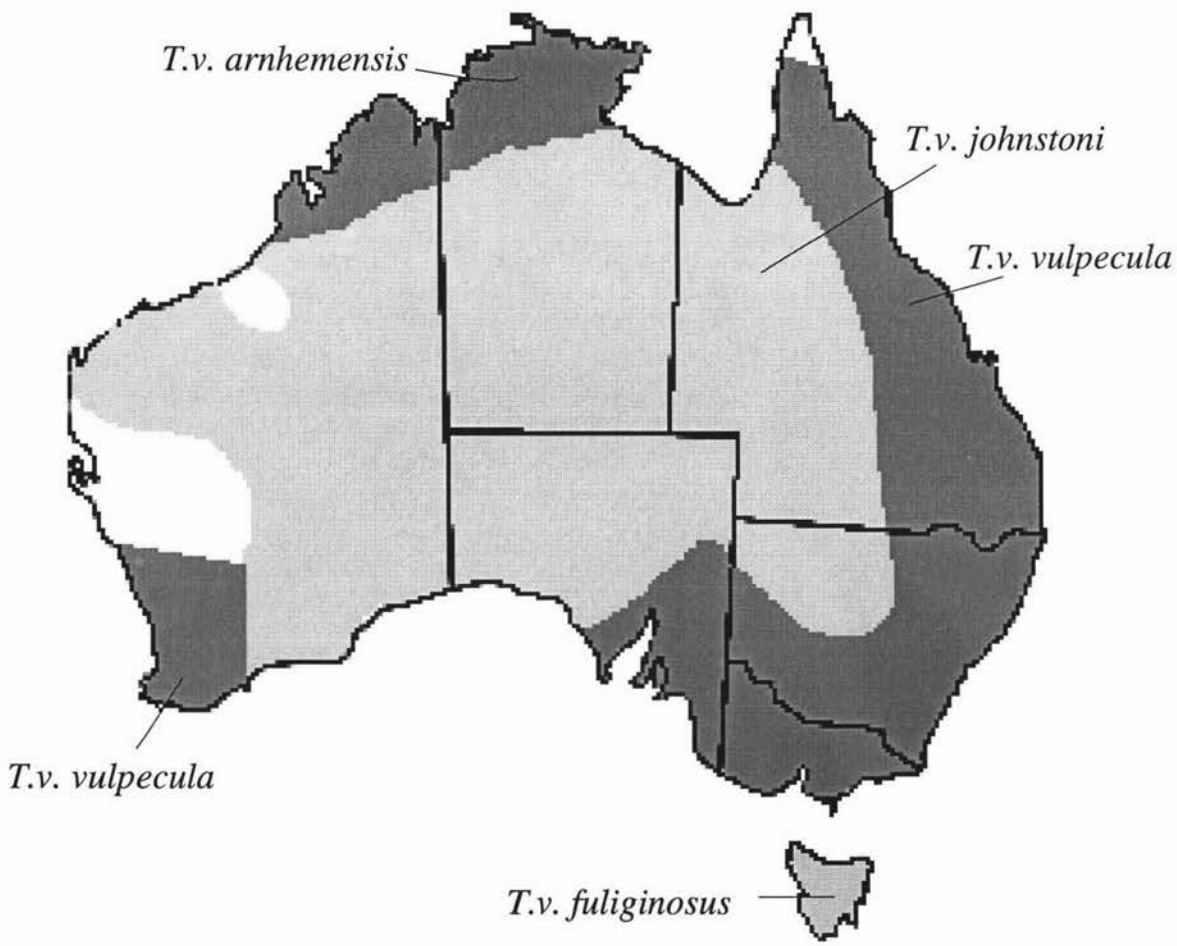


Figure 2.1 Map of Australia showing the distribution of *Trichosurus vulpecula*, and the proposed subspecific classifications.

2.1.2 Possums in New Zealand

Possums were introduced to New Zealand from Tasmania and Victoria, and possibly New South Wales (Pracy 1974; Triggs 1987). Victoria (and potentially New South Wales) was the predominant source of small grey possums. While Tasmania has both black and grey possums, it was chosen as a source for the valuable large black possums occupying the wetter parts of this state. Grey possums may also have been introduced from Tasmania, and it is possible that some black possums came from Victoria or New South Wales, where they occur in low proportions.

Possums were actively introduced to New Zealand from Australia from 1858 until 1924, although illegal introductions probably continued into the 1940's (Pracy 1974). For most of the introduction period, Tasmania was preferred as the source for possums, because the black and brown pelts were worth considerably more on the fur market. The bulk of the importations of Tasmanian blacks were released in the Westland, Grey, and Buller districts (Pracy 1974). From 1915 onwards, the introduction of further grey coloured possums was advocated, as a means of improving the breed. The value of black, brown and red-brown skins was declining, and there was a corresponding demand for grey possums by the fur industry (Pracy 1974).

Thirty-seven separate introductions were made from Australia, and locally established animals were released again in at least a further 402 locations (Pracy 1974, Brockie 1992). This was accomplished generally by acclimatisation societies, and occurred in new and already colonised areas, with the aim of "improving" the colour or size of the breed. Exact numbers, and relative proportions of each colour morph released in each area are largely unknown. The result was extensive mixing of Tasmanian and mainland Australian types, along with a rate of colonisation of New Zealand that far outstripped that which would have occurred by natural means alone. After this initial period of artificial introduction and spread, there has been a period of natural colonisation resulting in a present day distribution of possums where less than 5% of New Zealand remains uncolonised (Figure 2.2). Consequently, most possum populations in New Zealand are mixed with respect to coat colour, having both black and grey individuals (Figure 2.3).

Figure 2.2 Diagram of the spread of the brushtail possum in New Zealand from 1858 to 1996. Modified from Landcare Research [online] http://www.landcare.cri.nz/science/possums

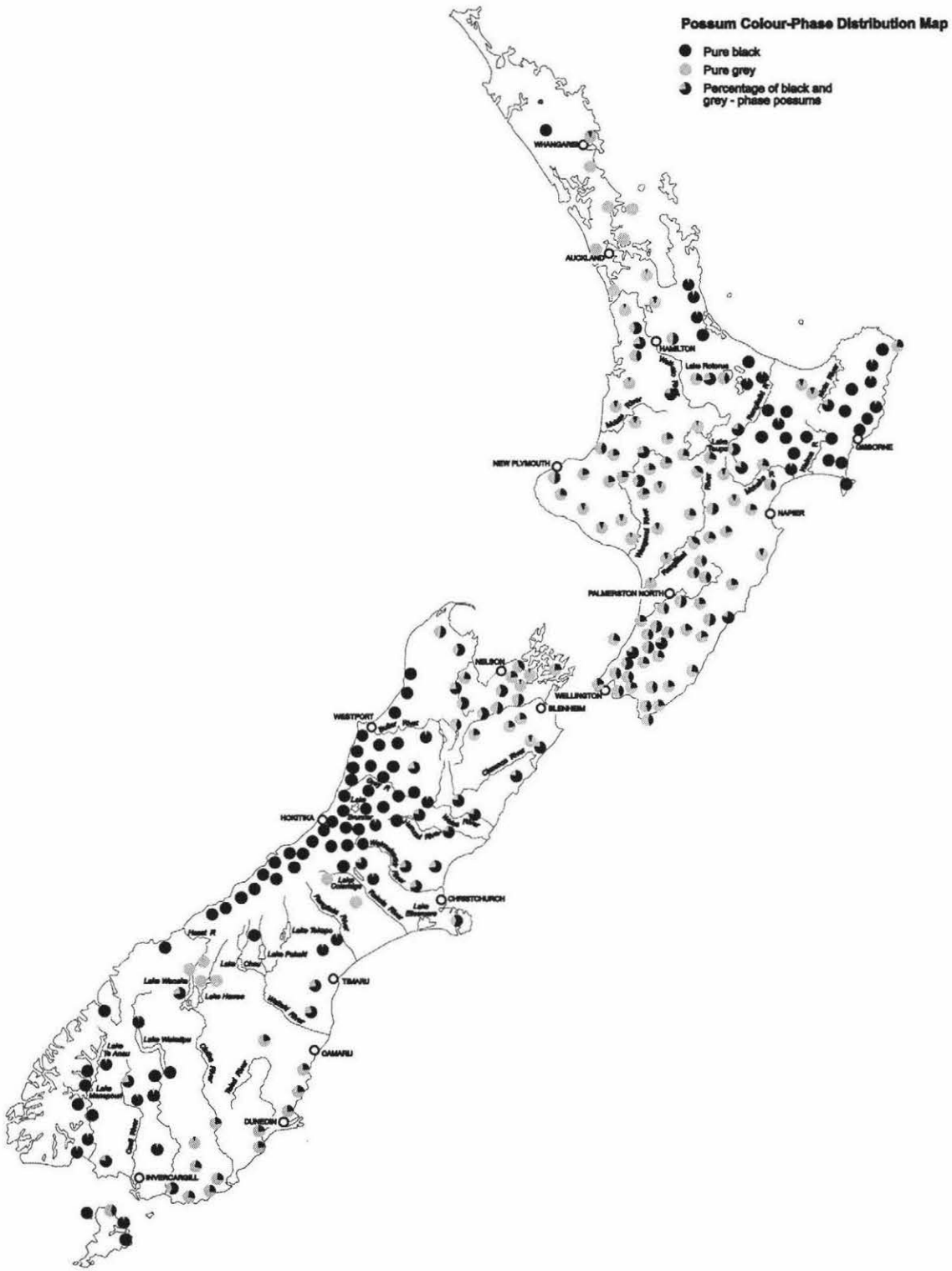


Figure 2.3 Map of New Zealand showing the percentage of each possum coat colour phase at selected sites. Taken from Pracy & Coleman (1998).

The only previous population genetic studies of New Zealand possums from throughout the country were conducted with allozymes (Triggs 1987; Triggs and Green 1989). Triggs and Green (1989) found that populations of predominantly black individuals were genetically most similar to Tasmanian possums, while predominantly grey populations were genetically closer to possums from Victoria and New South Wales.

An analysis of the DNA sequence variation inherent in the mitochondrial DNA control region and the cytochrome *b* gene of brushtail possums found very little variation in the cytochrome *b* gene, but higher levels of variation in the mtDNA control region (Aitken 1997). Only two individuals were compared, both from the Tararua Ranges. Thus, the assessed levels of sequence variation could be considered the minimum likely to occur in New Zealand possums. Broadening this survey by directly sequencing the most variable portion of the mitochondrial genome - the mtDNA control region - in a large number of individuals from throughout New Zealand, should allow a greater understanding of the genetic variation inherent in possum populations, and whether this variation is structured with respect to geographic location of the samples, or with morphological characteristics. Using phylogenetic analyses of the genetic variation in New Zealand and Australian possums, and comparing these data to the earlier allozyme results may allow a greater understanding of the patterns of population genetic variation and maternal origins of possums in New Zealand.

The aims of the research described in this chapter were as follows:

- To assess the levels of mtDNA variation in possums in New Zealand, and from that information develop mtDNA population markers effective for all possums in New Zealand.
- To identify any broad-scale genetic structure in possums in New Zealand, which might provide new information on possum ecology.
- To compare levels of variation in New Zealand possums with those in Australian possums, and attempt to identify potential maternal origins of New Zealand possums.

2.2 Materials and Methods

Recipes for all stock solutions and buffers are provided in Appendix One.

2.2.1 Study populations and DNA extractions

Tissue samples (ear or liver) were collected from 70 brushtail possums from 14 sites (Figure 2.4, Table 2.1) designed to encompass as widely as possible the geographic distribution of possums in New Zealand. Blood was obtained from two localities in Australia: Tasmania, which is known to have been a source of New Zealand possums, and South Australia, which has never been implicated as a source of possums for New Zealand. It was not possible to obtain genetic material from the other Australian states from which New Zealand possums were sourced. (Figure 2.5, Table 2.1).

Tissue samples were stored at -80°C or in ethanol until DNA extraction using standard phenol/chloroform procedures, as outlined in Sambrook *et al.* (1989) with some modifications.

To extract DNA from ear or liver samples, tissue was homogenised with a scalpel and transferred to an eppendorf tube containing 400 µl SET buffer, 50 µl 10% SDS and 50 µl proteinase-K (20 mg/ml). Incubation overnight with constant rotation at 55°C ensured complete digestion of the tissue. If the tissue had been stored in ethanol, 50 µl of 5 M NaCl was then added. Protein and cell debris were removed from the solution by adding an equal volume (500 µl) of phenol. The tube was rocked for 30 minutes, then centrifuged for 5 minutes at 13,800 *g*. The aqueous phase (containing the DNA) was removed to a fresh tube with an equal volume (450 µl) of phenol: chloroform: isoamyl alcohol (PCI, 25:24:1). The rocking and centrifugation steps were repeated, and the interface and organic (bottom) layer removed. This PCI step was then repeated. An equal volume of chloroform: isoamyl alcohol (CI, 24:1) was added, the rocking and centrifugation steps repeated, and the organic (bottom) layer removed. This CI step was then repeated. The DNA was precipitated by adding 60 µl of 3 M NaOAc pH 5.2, and 1 ml of ice-cold ethanol, and rocking for 30 minutes. Spooled DNA was removed with a pipette tip, placed in 1 ml 70% ethanol, and spun for 30 minutes at 13,800 *g*.

Figure 2.4 Map of New Zealand showing the sites from which possum tissue was collected.

Figure 2.5 Map of southern Australia, showing locations from which blood was collected.

Table 2.1 Sampling locations and sample sizes for possum tissue collected in Australia and New Zealand. Site names are used throughout the text to identify each population.

SITE NAME	LOCATION	MAP REFERENCE	SAMPLE SIZE
	Australia		
Launceston	Tasmania	41° 25′ 147° 07′	5
Adelaide	South Australia	34° 56′ 138° 36′	3
Kangaroo Island	South Australia	35° 50′ 137° 06′	3
	New Zealand		
Coatesville	Auckland	36° 43′ 174° 38′	4
Huapai	Auckland	36° 46′ 174° 32′	4
Hohotaka	near Taumarunui	38° 55′ 175° 26′	5
Tararua Ranges	near Levin	40° 50′ 175° 22′	7
Orongorongo Valley	Wellington	41° 25′ 174° 54′	5
Cape Palliser	South Wairarapa	41° 37′ 175° 15′	5
Paponga	Golden Bay	35° 18′ 173° 27′	5
Kumara	Westland	42° 38′ 171° 11′	5
Amuri Range	North Canterbury	42° 31′ 172° 11′	5
Ashley Gorge	Canterbury	43° 14′ 172° 13′	5
Banks Peninsula	Canterbury	43° 44′ 172° 52′	5
Longwood	Southland	46° 21′ 167° 50′	5
Halfmoon Bay	Stewart Island	46° 54′ 168° 09′	5
Long Beach	Chatham Island	44° 50′ 176° 34′	5

If there was insufficient DNA to spool, the sample was spun for 30 minutes at 13,800 *g* and the supernatant removed. Spooled and unspooled samples were then washed (once for spooled and twice for unspooled) in 1 ml of 70% ethanol, and spun for 30 minutes at 13,800 *g*. Finally the extracted DNA was resuspended in 40 µl of water. Dilutions of one in one hundred were made for use in PCR reactions. Visualisation on an agarose gel showed that this protocol yielded high molecular weight genomic DNA (~20 kb). A detailed protocol is provided in Appendix Two.

To extract DNA from blood, ice-cold lysis solution was added to the blood to a total volume of ~25 ml and mixed gently. The solution was left on ice for 30 minutes, then centrifuged at 4°C for 15 minutes at 4750 *g*. The supernatant was discarded and the pellet rinsed two or three times with small volumes of lysis solution, to remove red blood cells. The pellet was then resuspended in 450 µl of resuspension buffer, vortexed, and mixed with 40 µl of 10% SDS and 20 µl of Proteinase-K (20 mg/ml). Incubation overnight with constant rotation at 55°C ensured complete digestion of the tissue. The protocol was then continued as outlined above, starting with the phenol step. A detailed protocol is provided in Appendix Two.

2.2.2 Primer design

Sequence for the entire mtDNA control region of *Trichosurus vulpecula* was determined by Aitken (1997), and this was used to design two sets of primers to amplify a total of ~940 bp of the control region, including the entire ETAS and central conserved domains. Primers were designed, where possible, to anneal to conserved sites and to have high melting temperatures (either through high GC content or through length). The two sets of primers are: Tv5'F 5'-GGACACATCAAGAAGAGAGGCA-3', Tv5'R 5'-GGATGGTGATCTCTCGTGAGGT-3', Tv3'F 5'-CACTAGCATATCAT-CACCAT-3', and Tv3'R 5'-TGTATCCCATATTATCACTT-3'. The primer pair Tv5'F + Tv5'R amplify ~570 bp of the ETAS domain at the 5' end of the control region, while Tv3'F + Tv3'R amplify ~430 bp of the central conserved domain and right variable domain. These two primers overlap by 63 bp, allowing a consensus sequence of ~940 bp for each individual to be obtained. The relative position and orientation of the primers within the mtDNA control region are shown in Figure 2.6.

Figure 2.6 Map of the mtDNA control region of possums showing the position of primers Tv5'F, Tv5'R, Tv3'F and Tv3'R (not drawn to scale). ETAS = extended termination associated sequence (after Sbisa *et al.* 1997), CSB = conserved sequence block.

2.2.3 Amplification of mtDNA control region sequences

Polymerase chain reaction (PCR) amplification of double-stranded DNA was performed in 25 µl volumes, containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), and 10-50 ng of template DNA. The typical thermocycling procedure consisted of an initial denaturing step of 94°C for 2 minutes, followed by 30 cycles of 94°C for 40 seconds, 56°C for 40 seconds and 72°C for 40 seconds, and a final extension step of 72°C for 4 minutes. The annealing temperature of 56°C was found to be optimal for both sets of primers. All PCR reactions were performed in a Hybaid Omn-E (Hybaid Ltd, UK).

2.2.4 Purification and quantification of amplified DNA

Double-stranded PCR products were purified prior to sequencing using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals) according to the manufacturers instructions. The DNA was eluted with 60 µl 1mM Tris-HCl, and this volume was reduced to 10 – 15 µl in an Eppendorf Concentrator 5301 (Eppendorf, Germany).

Purified DNA was quantified visually on an agarose gel. For each sample, 1 µl of purified product was mixed with 2 µl of loading buffer containing xylene cyanol, and 2 µl of water, and loaded on a 0.8% agarose gel containing 2 µl of ethidium bromide. A low molecular mass ruler (Bio-Rad, CA) was loaded alongside the samples for quantifying the DNA. The samples were electrophoresed in a minigel chamber (Horizon®58 GibcoBRL, MD) with 1X TBE buffer at 100 V for 20-30 minutes. Products were visualised by UV trans-illumination and recorded via a gel documentation system (Insta Doc™ System, Bio-Rad, CA).

2.2.5 Sequencing of purified PCR products

The primer pairs Tv5'F + Tv5'R and Tv3'F + Tv3'R were used to obtain mtDNA control region sequence for 70 possums. The forward primers (Tv5'F and Tv3'F) were used as sequencing primers, and, where necessary, the reverse primers were also used to increase the amount of readable sequence, and to confirm each new haplotype. Purified DNA was used as template for ABI PRISM® sequencing reactions. Reactions were performed in 10 µl volumes containing 4 µl ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, CA.), 3.2 µl of either the forward or reverse primer (10 pM/µl), 10-15 ng of purified PCR product, and water, if necessary, to make up to volume. Reactions were completed in a Hybaid Omn-E thermal cycler (Hybaid Ltd, UK) with the following thermocycling profile: 25 cycles of 96°C for 30 seconds, 56°C for 15 seconds and 60°C for 4 minutes.

Precipitating twice in isopropanol (60% final concentration), and pelleting at 16,000 *g* for 20 minutes (first wash) and 5 minutes (second wash) purified the extension products. The sample was dried under vacuum for 5 minutes before being stored at -20°C until sequenced according to the manufacturer's recommendations, in an ABI 377 automated DNA sequencing system (Perkin-Elmer Applied Biosystems, CA) at the Massey University sequencing facility (MuSeq) and the Waikato DNA Sequencing Facility, Waikato University.

2.2.6 Analysis

OLIGO 4.0-s (National Biosciences, Inc) and Amplify 1.2 (University of Wisconsin, WI) were used as aids to design the primers. DNA sequences were aligned, checked by eye, and edited using Sequencher™ 3.1.1 (Gene Codes, Corp).

Phylogenetic analyses were made using MEGA 2.0 (Kumar *et al.* 2001) to analyse the relationships between haplotypes. Two types of tree-building methods were used: neighbour-joining (NJ, Saitou and Nei 1987) and maximum parsimony (MP, Fitch 1971). For NJ trees, the Jukes-Cantor distance (Jukes and Cantor 1969) was used. For MP trees, the branch and bound method was used to search for the most parsimonious trees. Tree topology for the NJ tree was tested using an interior branch test (Rzhetsky and Nei 1992) and a bootstrap test (Falsenstein 1985) with one thousand replications;

and a bootstrap consensus tree (x 1000 replications) was constructed for the MP tree. A 50% majority-rule tree of all most parsimonious trees also was constructed.

Traditional methods of phylogeny reconstruction, using methods such as NJ and MP, make assumptions that may be invalid at the population level, such as the assumption that ancestral haplotypes are no longer present in the population. Coalescent theory predicts that the ancestral haplotypes will actually be the most frequent sequences found in a population level survey (Clement *et al.* 2000 and references therein). Another way of representing genealogical relationships among sequences at the population level is to use network representations, which enable the incorporation of genealogical information associated with population level divergences. For this reason, parsimony networks of haplotypes (Program TCS 1.13, Clement *et al.* 2000) were obtained.

2.3 Results

2.3.1 Sequence variation and diversity of mtDNA control region haplotypes of possums from throughout New Zealand

The analysis of sequence variation among 70 individuals from 14 locations identified a total of 35 nucleotide substitutions and a 1 bp insertion/deletion (indel) that together define nine haplotypes differing at one or more nucleotide sites (mean 15, range 1-24, Table 2.2). The indel was the first variable site, and occurred near the beginning of the readable sequence for each haplotype. Thus, for consistency and ease of numbering, all sequence before the indel was discarded, and numbering begun with the indel at position one. The most common haplotype (designated haplotype 6) was found in 47 of the 70 possums (67%) and in 11 of the 14 (79%) populations that were surveyed (Figure 2.7, Table 2.3).

Transitions (T-C, 49% A-G, 45%) far outweighed transversions (C-A, 3%, A-T, 3%). This is consistent with the bias towards transitions reported for other intraspecific mtDNA comparisons (Brown *et al.* 1982; Greenberg *et al.* 1983; Brown *et al.* 1986; Norman *et al.* 1994). There was also a marked A-T bias in the sequences (A-T, 68%,

Table 2.2 Polymorphic nucleotide sites in the mtDNA control region of *Trichosurus vulpecula* in New Zealand. Dots indicate that the base is identical to the uppermost consensus sequence. The colon (:) indicates that at this position the base is missing in these sequences (an indel).

					1	1	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	4	4	4	5	5	6	7	8	8	8	8	8	8
			3	6	0	1	2	9	1	5	5	5	6	6	7	0	1	2	2	3	6	8	8	8	2	3	6	1	9	7	8	0	1	1	3	3	3
Haplotype	n	1	3	9	9	7	7	9	2	0	4	5	4	8	6	9	1	0	5	6	3	0	6	7	6	3	0	3	6	7	8	5	7	9	6	7	9
Consensus		:	C	T	T	G	A	C	T	T	G	T	T	A	C	A	A	C	A	C	C	T	A	A	C	C	A	T	G	T	A	A	A	C	A	A	A
1	1	C	.	.	C	.	.	T	.	.	A	C	.	.	T	G	.	.	G	.	T	.	G	.	.	.	.	.	A	.	G	.	G	T	.	T	.
2	8	C	.	C	C	.	.	.	.	.	A	C	.	G	.	G	.	.	G	.	.	.	G	.	.	.	G	.	A	.	.	.	G	.	.	T	.
3	1	C	A	.	.	A	G	.	.	C	.	.	.	G	T	.	G	.	.	T	T	C	.	.	T	T	.	C	A	C	.	G	.	.	.	T	G
4	5	C	A	.	.	A	G	.	.	C	.	.	.	G	T	.	G	.	.	T	T	C	.	.	T	T	.	C	A	.	.	G	.	.	.	T	G
5	1	.	.	.	.	.	.	T	.	.	A	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	37	.	.	.	.	.	.	T	.	T	A	.	C	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
7	3	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
8	3	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.
9	1	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.

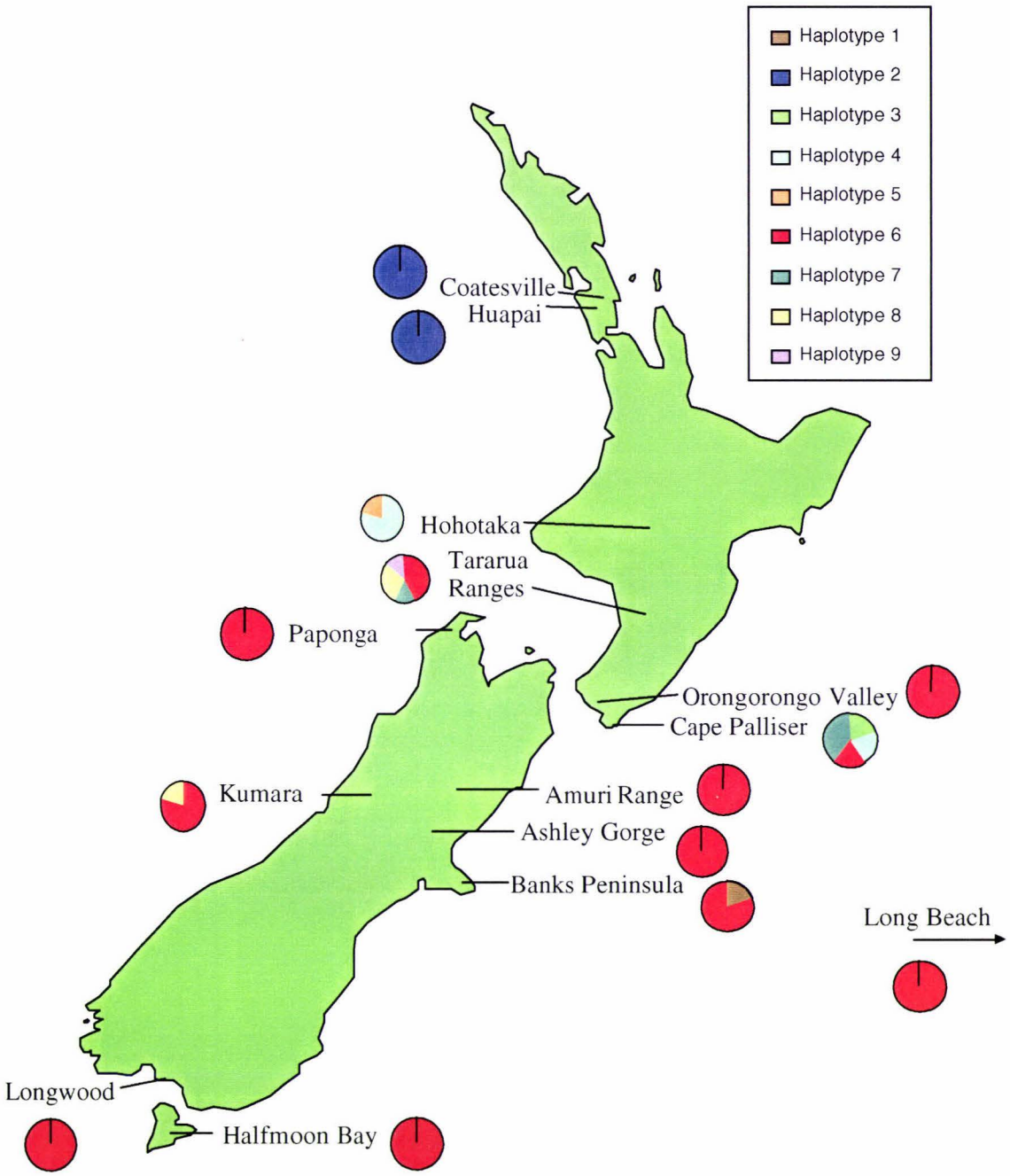


Figure 2.7 Map of New Zealand with pie graphs of haplotype frequencies at each site sampled.

Table 2.3 Haplotype frequencies for the 14 *Trichosurus vulpecula* populations in New Zealand. Population localities are shown in Figure 2.4. Haplotypes are numbered, with their frequency within populations in parentheses. *n* is the number of individuals sampled.

Population	*n*	Haplotype (frequency)
Coatesville	4	2 (1.00)
Huapai	4	2 (1.00)
Hohotaka	5	4 (0.80), 5 (0.20)
Tararua Ranges	7	6 (0.43), 7 (0.14), 8 (0.29), 9 (0.14)
Orongorongo Valley	5	6 (1.00)
Cape Palliser	5	3 (0.20), 4 (0.20), 6 (0.20), 7 (0.40)
Paponga	5	6 (1.00)
Kumara	5	6 (0.80), 8 (0.20)
Amuri Range	5	6 (1.00)
Ashley Gorge	5	6 (1.00)
Banks Peninsula	5	1 (0.20), 6 (0.80)
Longwood	5	6 (1.00)
Halfmoon Bay	5	6 (1.00)
Long Beach	5	6 (1.00)

G-C, 32%), and low overall G content (14.1%), both of which have been recognised as general features of mtDNA control region sequences (Avise *et al.* 1987; Moritz *et al.* 1987). The overall base compositions were A, 35.2%; C, 17.5%; G, 14.1 %; T, 33.2 %.

The average pairwise distance (nucleotide diversity, d, Nei 1987) among all individuals in the sample was 1.0% (SD = 0.52%), while the overall haplotype diversity (h; Nei 1987), i.e. the probability that two possums chosen at random from throughout New Zealand have different haplotypes, was 59.9% (SD = 6.8%). A discovery curve showing the detection of new haplotypes with increasing number of individuals sampled is shown in Figure 2.8. On average, after 85% of the individuals had been sampled, no new haplotypes were discovered. This suggests that it is likely that all, or nearly all, of the haplotypes present in New Zealand were detected (although see section 2.4.1). Most populations sampled contained both black and grey individuals (Figure 2.9).

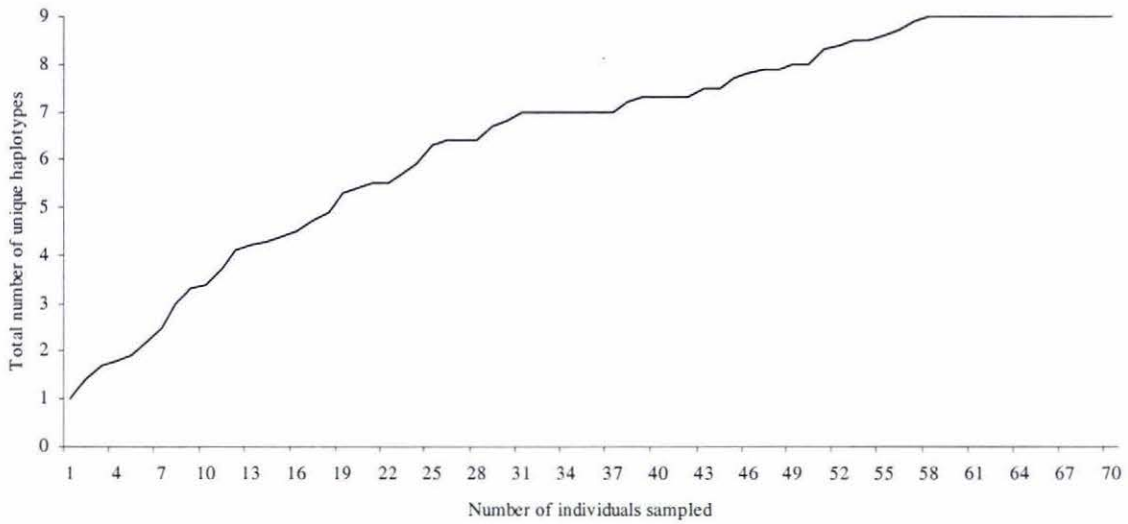


Figure 2.8 Discovery curve for the number of mtDNA control region haplotypes in New Zealand *Trichosurus vulpecula*, showing cumulative number of new haplotypes detected with increasing number of individuals sampled, with random addition of new individuals. Ten replicates were conducted, and the average is shown.

Jukes-Cantor distances ranged from 0.001 to 0.028 within New Zealand samples (Table 2.4). Parsimony analysis found seven most parsimonious trees of 44 steps. The two methods of phylogenetic reconstruction showed similar topologies, each identifying three clades, suggesting that the phylogenetic reconstruction was fairly robust to the type of method used (Figure 2.10a&b). The distance matrix (Table 2.4) shows that clades a and b are the most closely related clades, and that clade c is roughly equidistant between them. Six of the nine haplotypes are represented by multiple individuals. Of those six, five have possums of both coat colours. The exception to this is haplotype 2, which only has grey possums. This haplotype groups with haplotype 1 to form a clade. This clade (clade b) has only grey possums, suggesting there is an association between coat colour and clade. This association, between clade and coat colour, is statistically significant ($\chi^2 = 12.34$, $p = 0.030$). The interaction between coat colour and haplotype however, is not statistically significant ($\chi^2 = 7.14$, $p = 0.982$). The small number of

individuals tested (70 overall) may mean that any associations in the data are caused by random sampling error.

The three clades are still apparent using a parsimony network analysis (Figure 2.11) but the network suggests that clade c is more closely related to clade a than it is to clade b, rather than being equi-distant between them as the Jukes-Cantor distance matrix suggested.

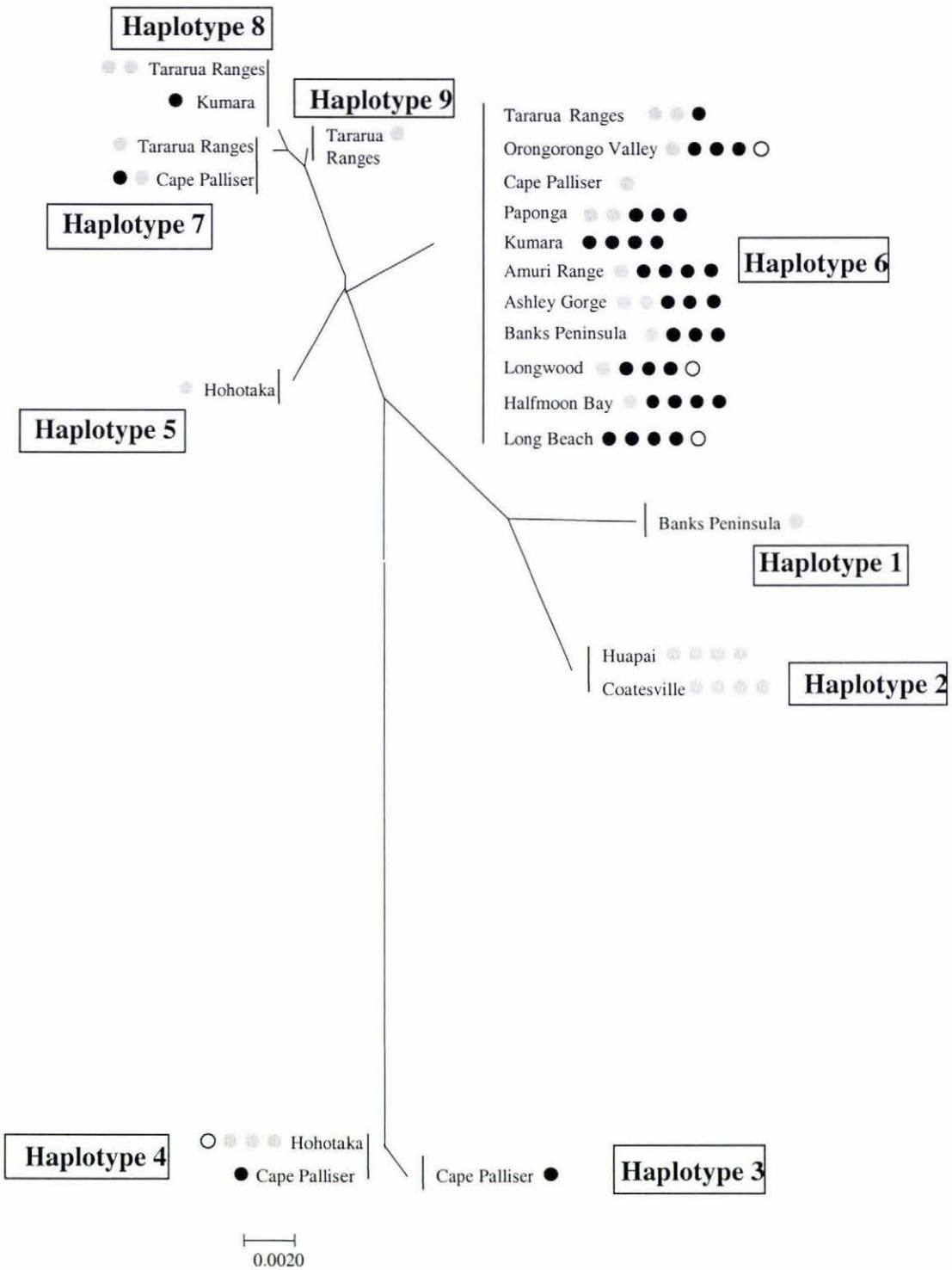
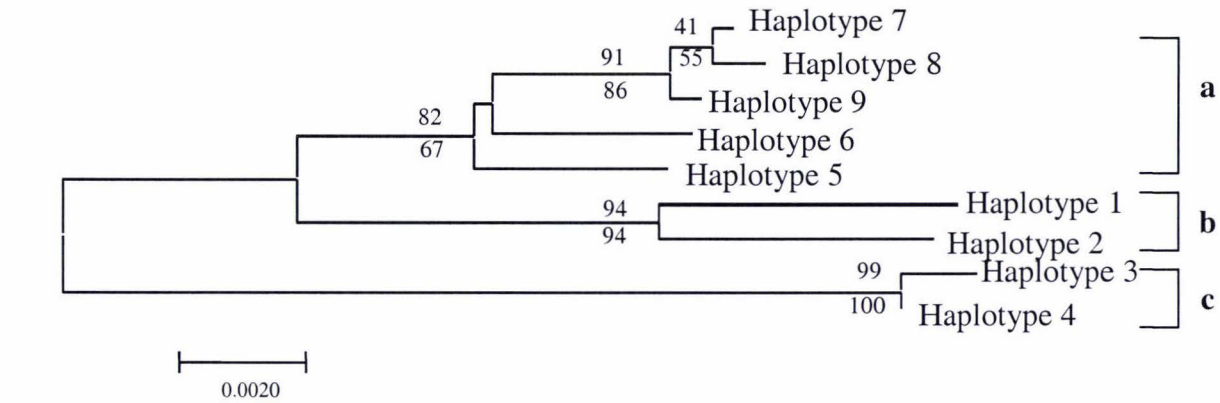


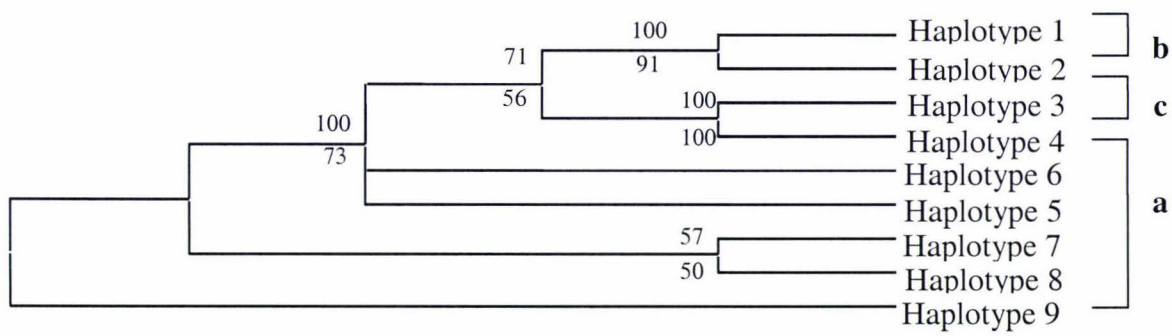
Figure 2.9 Unrooted NJ tree showing the nine unique mtDNA haplotypes found in the *Trichosurus vulpecula* individuals sampled in New Zealand. Sites names, indicating where possums with each haplotype originated from, are given. Circles next to each site name represent individual possums from that site. Grey circles (●) represent possums with grey fur, black circles (●) represent black furred possums, and open circles (○) represent possums for which coat colour was unrecorded or unable to be categorised. Haplotypes are numbered 1 – 9 consistently throughout the thesis. Branch lengths represent genetic distance based on the Jukes-Cantor correction (Table 2.4).

Table 2.4 Pairwise distance comparisons between all haplotypes using Jukes-Cantor measure of distance.

Clade	b		c		a											
Haplotype	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Tas1**	**Tas2**	**Tas3**	**Tas4**	**Ade1**	**Ade2**	**Kang Is**
1	-															
2	0.009	-														
3	0.029	0.029	-													
4	0.027	0.027	0.001	-												
5	0.014	0.014	0.026	0.025	-											
6	0.015	0.018	0.025	0.024	0.006	-										
7	0.019	0.017	0.024	0.023	0.007	0.008	-									
8	0.020	0.018	0.025	0.024	0.008	0.007	0.001	-								
9	0.019	0.017	0.024	0.023	0.007	0.006	0.002	0.001	-							
Tas1	0.019	0.021	0.029	0.027	0.009	0.011	0.009	0.008	0.009	-						
Tas2	0.017	0.017	0.024	0.023	0.007	0.008	0.009	0.008	0.009	0.009	-					
Tas3	0.019	0.019	0.026	0.025	0.009	0.011	0.012	0.011	0.012	0.012	0.002	-				
Tas4	0.017	0.017	0.024	0.023	0.002	0.006	0.005	0.006	0.005	0.009	0.009	0.012	-			
Ade1	0.046	0.043	0.046	0.045	0.038	0.040	0.038	0.037	0.038	0.038	0.038	0.038	0.038	-		
Ade2	0.046	0.051	0.053	0.052	0.046	0.045	0.051	0.050	0.048	0.043	0.048	0.046	0.046	0.024	-	
Kang Is	0.042	0.050	0.052	0.051	0.045	0.043	0.047	0.046	0.045	0.042	0.045	0.042	0.045	0.032	0.032	-



a



b

Figure 2.10 Unrooted phylogenetic reconstructions among nine mtDNA haplotypes of *Trichosurus vulpecula* in New Zealand based on 880 nucleotides of mtDNA control region. a: Neighbour-joining tree estimated from Jukes-Cantor distances. The numbers above the branches indicate the interior branch test support, and numbers below the branches indicate the bootstrap support. In both cases, support is only indicated at nodes if found in more than 50% of 1000 replicates. b: Maximum-parsimony tree. The tree shown is the consensus of seven most-parsimonious trees resulting from a branch and bound search of the nine haplotypes. Tree length = 44 steps, CI = 0.80, RI = 0.79. The numbers above the branches indicate the 50% majority rule consensus, and numbers below the branches indicate the consensus tree bootstrap support. In both cases, support is only indicated at nodes if found in more than 50% of 1000 replicates. In both reconstructions, it is possible to identify three clades, a, b, and c.

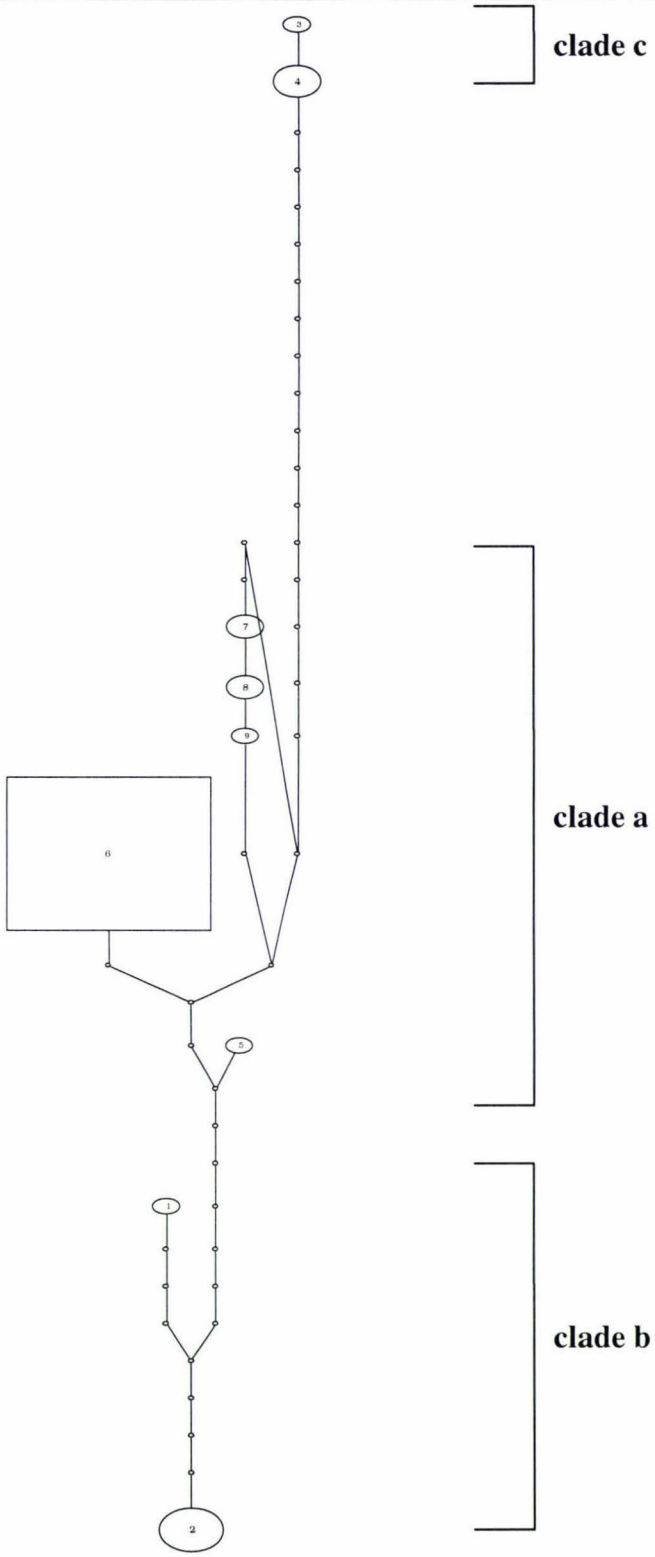


Figure 2.11 Parsimony network of the nine mtDNA control region haplotypes found in *Trichosurus vulpecula* in New Zealand. Size of the square or ovals corresponds to the haplotype frequency. The haplotype with the highest outgroup probability is shown as a square (haplotype 6). Dots on connecting branches represent intervening mutational steps between haplotypes.

2.3.2 Relationship between New Zealand and Australian possums

Nucleotide sequence of 11 possums from Australia revealed six haplotypes, none of which were found in New Zealand possums. Jukes-Cantor distances between New Zealand and Australian possums ranged from 0.002 - 0.053 (Table 2.4). Phylogenetic reconstruction groups the four Tasmanian haplotypes with the haplotypes in clade a, which included the most common New Zealand haplotype (haplotype 6; Figures 2.12 and 2.13). Indeed, haplotype 5 is more closely related to a Tasmanian haplotype (Tasmania4, Jukes-Cantor distance = 0.002) than to any of the New Zealand haplotypes (Jukes-Cantor distances = 0.006 - 0.026), in all three analyses. New Zealand and Tasmanian possums range in distance from 0.002 - 0.029 (Table 2.4).

The six South Australian possums have three haplotypes, all of which are very different from the haplotypes found in New Zealand, and form a separate clade in the NJ tree. New Zealand and South Australian possums range in Jukes-Cantor distance from 0.038 - 0.053 (Table 2.4, Figures 2.12 and 2.13). This separate South Australia clade acts in a similar way as an outgroup, and causes a change in the topology of the MP tree. Before the Australian haplotypes were added, the grouping of clades a and b was not well supported statistically, and occurred in five of the seven (71%) most parsimonious trees. When the Australian haplotypes were added, the grouping of clades a and b is still not particularly well supported statistically, but it occurs in 100% of the 42 most parsimonious trees. The topologies of the neighbour-joining and maximum parsimony trees are very similar once the Australia samples are added. In both reconstructions, the New Zealand and Tasmanian haplotypes form a monophyletic group with high statistical support (99 - 100%, Figure 2.12a and b). The three South Australian haplotypes are separated from this monophyletic group by long branches. In the parsimony network (Figure 2.13), the South Australian lineages are so different from the New Zealand and Tasmanian possums (representing the monophyletic grouping found with NJ and MP) that they do not join the main network, even when 20 mutational steps were allowed for. Furthermore, the Adelaide and Kangaroo Island haplotypes did not join together, showing that these two haplotypes are more than 20 mutational steps apart.

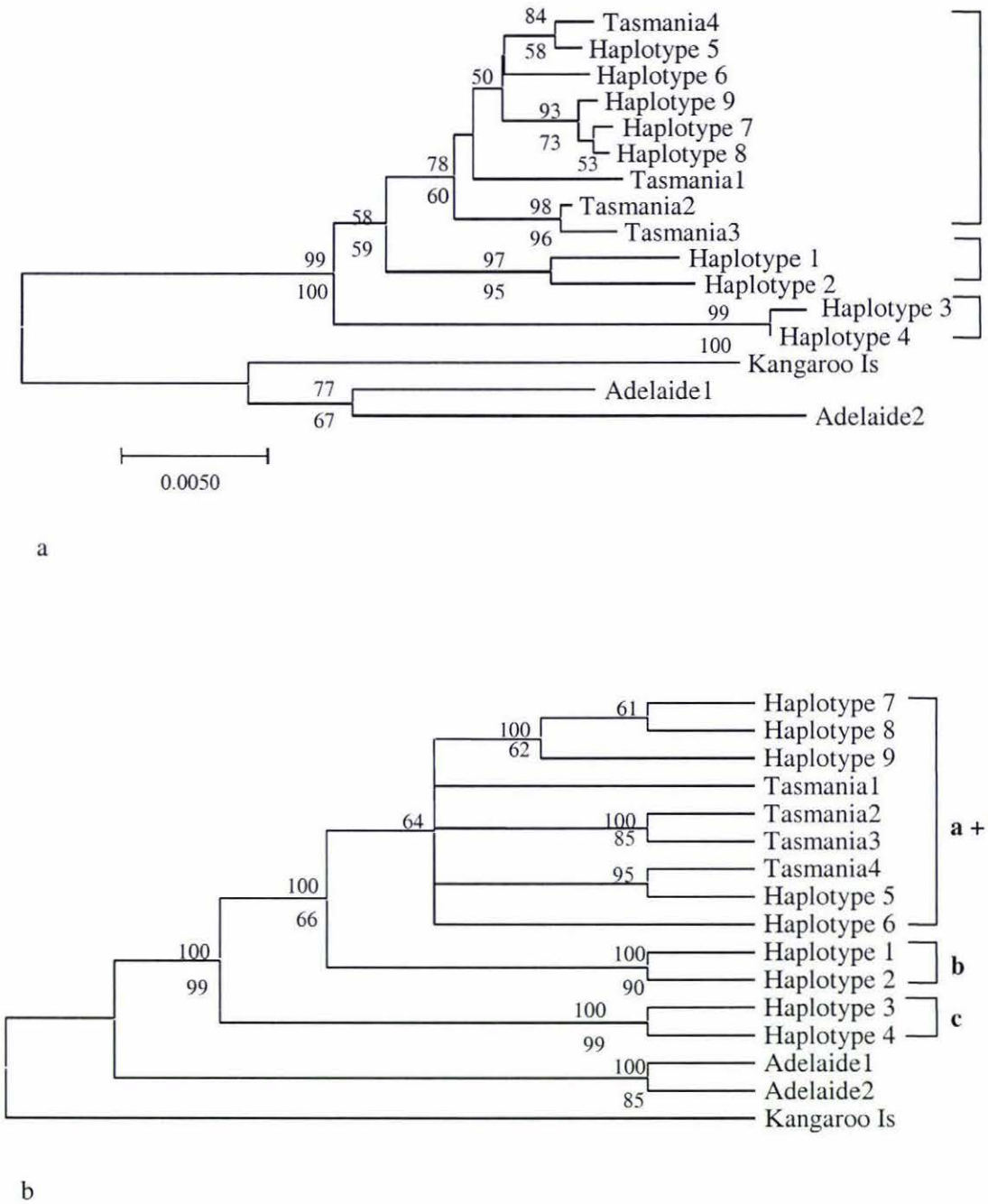


Figure 2.12 Phylogenetic trees of mtDNA haplotypes of *Trichosurus vulpecula* in Australia and New Zealand. a: mid-point rooted neighbour-joining tree estimated from Jukes-Cantor distances. The numbers above the branches indicate the interior branch test support, and numbers below the branches indicate the bootstrap support. In both cases, support is only indicated at nodes if found in more than 50% of 1000 replicates. b: unrooted maximum-parsimony tree. The tree shown is the consensus of 42 most-parsimonious trees resulting from a branch and bound search of the 16 haplotypes. Tree length = 113 steps, CI = 0.66, RI = 0.72. The numbers above the branches indicate the 50% majority rule consensus, and numbers below the branches indicate the consensus bootstrap support (x 1000 replicates). In both cases, support is only indicated at nodes if greater than 50%.

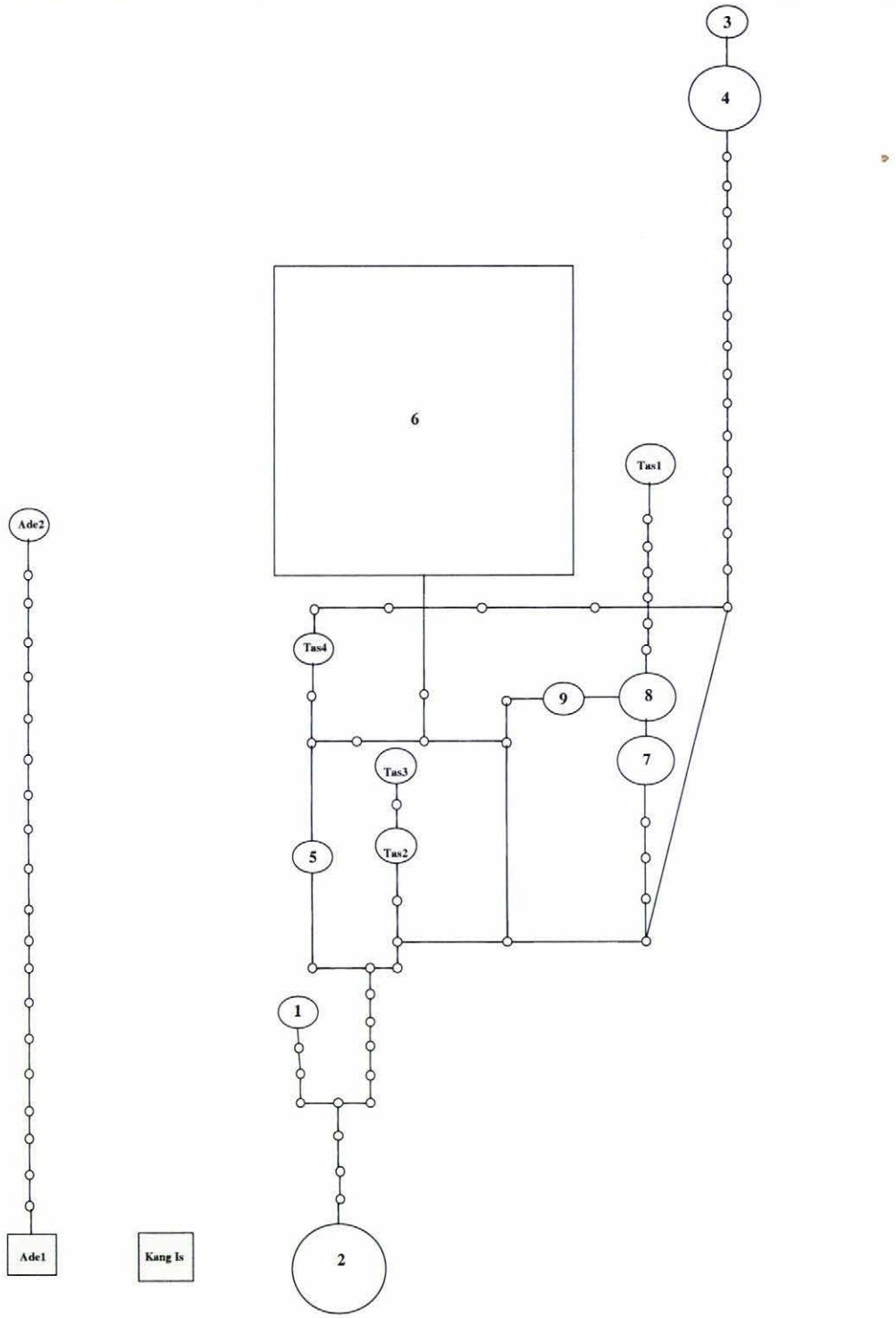


Figure 2.13 Parsimony network of the 16 mtDNA control region haplotypes found in *Trichosurus vulpecula* in Australia and New Zealand. Size of the square or ovals corresponds to the haplotype frequency. The haplotype with the highest outgroup probability is shown as a square. Dots on connecting branches represent intervening mutational steps between haplotypes. Twenty mutational steps were allowed for, thus unconnected haplotypes and networks are more than 20 mutational steps apart and cannot be connected. Numbers 1-9 correspond to haplotypes 1-9 respectively. Tas = Tasmania, Kang Is = Kangaroo Island, Ade = Adelaide. Australian haplotypes are shaded grey.

2.4 Discussion

2.4.1 Levels of mtDNA variation in New Zealand

The amount of variation in the mtDNA control region of brushtail possums throughout New Zealand seems quite low. There were only nine haplotypes identified in New Zealand. This is probably close to the actual number of mtDNA haplotypes, based on the discovery curve and because sampling occurred throughout New Zealand. However, some localities (such as Tararua Ranges and Cape Palliser) had almost as many haplotypes as the number of individuals sampled, including lineages unique to these areas, (e.g. haplotypes 3 and 9), so there may be some undiscovered haplotypes in these areas.

The overall paucity of variation suggests that possums have undergone substantial population bottlenecks during their introduction to New Zealand. The number of females originally introduced to New Zealand was likely between 100 and 150 (given that between 200 and 300 possums in total were introduced; Pracy 1974), yet all ~60 - 70 million possums found in New Zealand today may be descended from as few as nine female mtDNA haplotypes. This estimate must, however, be qualified for two reasons. Firstly, not all haplotypes are likely to have been detected, as mentioned above. If so, the number of founding mitochondrial lineages would be greater than nine. Secondly, two haplotypes were found in single individuals only, and differed from the next most genetically similar haplotype by only one base pair. These two rare haplotypes (and possibly others) may have arisen (via mutation and random drift) after possums were introduced to New Zealand, and persist at low levels. For instance, haplotype 3 is only found in one individual from Cape Palliser, and differs from haplotype 4 by one a single base pair mutation (Table 2.2). The parsimony network shows that haplotype 4 is ancestral to 3, because it is more closely related to the other New Zealand haplotypes. Thus, haplotype 3 may have arisen by mutation since possums were introduced to New Zealand. Given this criteria (low frequency of occurrence and similarity to more common haplotypes) haplotypes 1 and 9, and possible others, are potential candidates for haplotypes that have arisen since possums were introduced to New Zealand. If so, the estimate of nine founding lineages will be an overestimate. Further sampling around New Zealand, and more extensive sampling of areas with high haplotypic diversity would help to resolve these issues.

The three clades (a, b and c) do not reflect the geographic origin of the possums within New Zealand. Two of the three clades (a and b) have North and South Island possums, and two haplotypes are present in possums from both of the main islands (haplotypes 6 and 8). The North Island has the most haplotypic diversity, having 8 of the 9 (89%) haplotypes, whereas only 3 haplotypes (33%) were observed among the South Island individuals sampled (Table 2.3). Only a single haplotype was observed in each of the Stewart Island and Chatham Island samples (haplotype 6 in both cases), suggesting even more profound bottleneck effects with small introductions to islands.

The low level of variation for a maternal marker such as mtDNA is not a surprising result, as many of the introductions were unsuccessful at establishing possums in a region (Pracy 1974), suggesting that often females died with little or no reproductive success. Also, as coalescent theory predicts, mtDNA is particularly susceptible to random lineage extinction, especially after severe population bottlenecks (Hudson 1990, Birky 1991). Furthermore, there may not have been a great deal of mtDNA variation present in the possums originally introduced. Triggs (1990) found that levels of genetic divergence (measured using allozymes) between Tasmanian and three mainland populations from New South Wales, Victoria and South Australia, were low relative to those commonly found between populations and subspecies of other mammals. This raises the possibility that *Trichosurus vulpecula* may be a species with fairly low levels of variation overall. An analysis of the population genetics of the brushtail possum in Australia using mtDNA and microsatellite markers would help to resolve this. This study with mtDNA markers suggests a higher level of variation in Australia than New Zealand: for the 12 Australian individuals there were six haplotypes found, whereas for 70 individuals in New Zealand only nine haplotypes were found. A lower level of variation in New Zealand than Australia would be entirely expected, as variation would have been lost during the introductory phase.

2.4.2 Genetic structuring of possums in New Zealand

There is little geographic structuring to the haplotypic diversity found in New Zealand. There is only one haplotype in the Auckland region, haplotype 2, and this haplotype does not occur anywhere else in New Zealand. Haplotype 5 occurs only at Hohotaka, in the central North Island. Haplotype 6 is the most common haplotype, occurring in 67%

of the possums sampled. It is the only haplotype found on Stewart and Chatham Islands, isolated by water barriers from immigration by mainland possums. Haplotype 6 occurs in every population from the Tararua Ranges south, but not in the central and northern North Island populations sampled. Together, these results suggest some geographic structuring with respect to populations in the top half of the North Island compared to populations in the rest of New Zealand. However, haplotype 2 (found in the Auckland region) groups most closely with haplotype 1, which is only found in the South Island, while haplotype 5 (found only at Hohotaka) groups most closely with haplotype 6, the most widely distributed lineage. As such, the geographic structuring in the top half of the North Island may just be a random artefact of the introductory phase, or of the sampling scheme.

Possums have only been present in New Zealand for 150 years. A total of 200 - 300 individuals were introduced to New Zealand from a limited number of localities in Australia. This occurred over the space of sixty years, with small numbers of individuals being introduced from different source populations to different sites throughout the country. The founding of possum populations from limited numbers of individuals, and the failure of some of these individuals to successfully establish, would have caused genetic bottlenecks, especially in the maternally inherited mtDNA, which has approximately one quarter the effective population size of nuclear loci (Harrison 1989). Furthermore, the cumulative effect of further founding events during the colonisation of new territory would enhance bottleneck effects. Since possums have become established in New Zealand, there has been large and rapid population expansion, both in number of possums and their geographic distribution (aided in part by human intervention). Currently there are an estimated 60 - 70 million possums in New Zealand (Montague 2000). If one accepts the suggested upper limit to the number of introductions (300; Pracy 1974), a generous estimate of intrinsic growth rate (r_m = 0.3, Barlow 1991b) and a conservative estimate of the population in the 1980s (69 million, Brockie 1992), then at least four decades of unrestricted population growth are required to reach a population of this size (Efford 2000). The continuing expansion of possums into new areas over this time period (Figure 2.2) suggests that this is not an unlikely scenario. Given this set of circumstances (genetic bottlenecks, rapid population expansion and short time since introduction), it is not surprising that there is little or no geographic structure to mtDNA control region variation in possum

populations in New Zealand. Genetic bottlenecks would have caused the random extinction of some mtDNA lineages, while rapid population expansion would mean that further extinction of lineages would be highly unlikely. In each population, lineage survival and extinction would occur at random. By chance the same lineage might be introduced and survive in two populations that are otherwise completely genetically isolated. Using a traditional estimate of gene flow, such as F_{ST} (Wright 1951), will produce the misleading result that these two populations are connected by gene flow. Complete lineage sorting will require random genetic drift and mutation to cause the divergence of genetically isolated populations. While this may be beginning to happen, there has been insufficient time for extensive geographic structuring to occur, let alone be detected. Boileau *et al.* (1992) suggest that rapid expansion of populations founded by a few individuals results in gene frequencies that are very resistant to change by gene exchange. If so, many more possum generations will be required before random lineage sorting has occurred to such a degree that population structuring will be apparent. Currently less than 75 possum generations are likely to have passed since their introduction, given that females generally begin successfully reproducing at two years, and the maximum time since introduction is 150 years, with some populations being much younger than this (Figure 2.2).

A more rapidly evolving marker may be more successful than mtDNA markers at detecting geographic structure in possums in New Zealand. For example, microsatellite markers, which evolve more rapidly than mtDNA sequence, might reveal structure obscured in mtDNA markers. However, because mtDNA may have only one quarter the effective population size of nuclear markers, mtDNA markers are good candidates for early detection of population structuring in females (Birky 1991).

2.4.3 Relationship between New Zealand and Australian possums

The nine haplotypes found in New Zealand were grouped into three clades, which suggests that there are three Australian origins of possums in New Zealand. The main clade of haplotypes (clade a), which contains 77% of all the possums analysed, is likely to be Tasmanian in origin, because all the possums from Launceston in Tasmania group within this cluster. Also, these haplotypes occurred in possums from throughout New Zealand (except for the Auckland region) and Tasmania was the main source of

possums during the majority of the introductory period (Pracy 1974). Auckland and Northland have only grey possums (Figure 2.3), and liberation records suggest that only grey possums from mainland Australia were released in these districts (Pracy 1974). The Auckland region has haplotype 2 only, which forms a clade with haplotype1, represented by a single possum from Banks Peninsula. Possums of both colours were released in Banks Peninsula. It is plausible that this clade is of Victorian or New South Wales origin. The remaining clade represented by haplotypes three and four may originate from the other of these two states. These possibilities could be resolved by direct sequencing of the mtDNA control region of possums from Victoria and New South Wales, and more extensive sampling of possums from Tasmania and New Zealand. A further analysis of variation with a nuclear marker (such as a microsatellite), or a paternal marker (such as the Y chromosome), will show whether the Tasmanian type has been particularly successful as a coloniser of New Zealand, or whether it is just female Tasmanian possums that are especially widespread.

Triggs and Green (1989) found that coat colour could be used as a rough estimator of the genetic origin of possums in New Zealand. While it did appear that there was some association between coat colour and clade, this association did not hold at the haplotypic level. The small number of individuals with some haplotypes means that any associations should be treated with caution until more extensive sampling can be conducted.

If the vast majority of possums in New Zealand today are Tasmanian in origin, then this has implication for possum control in this country. At low temperatures Tasmanian possums are more resistant to 1080 poison than possums from the mainland of Australia (discussed in Triggs 1987 and Triggs and Green 1989). This raises the question of whether dosages of 1080 will need to be increased to ensure a lethal dose is administered, as New Zealand has a similar climate to Tasmania. More research in this area is surely required.

The extreme dissimilarity of the South Australian possums from any of the mtDNA lineages found in New Zealand substantiates historical records (Thomson 1922; Wodzicki 1950; Pracy 1974) that South Australia was not a source of female brushtail possums for New Zealand. The inclusion of these individuals in phylogenetic analyses

shows that the *Trichosurus vulpecula* lineages in New Zealand are more similar to each other than any are to South Australian *Trichosurus vulpecula* lineages. If clades b and c are Victoria and New South Welsh in origin, or if both are Victorian, then these lineages are more similar to the putative Tasmanian lineages, than any are to South Australian lineages. Triggs (1990) also found that that Tasmanian possums differed less genetically from Victoria (Melbourne) and New South Wales (Sydney) possums than New South Wales possums did from South Australian (Adelaide) possums. This confirms the finding that South Australia was not a source of any possums for New Zealand.

Kerle *et al.* (1991) suggested the retention of Tasmanian possums as a separate subspecies, *Trichosurus vulpecula fuliginosus*, but did not support the classification of possums from New South Wales, Victoria and South Australia as separate subspecies, instead classifying them all simply as *T. vulpecula*. The current results suggest that, on the basis of mtDNA variation only, either South Australian populations also deserve subspecies status, or the Tasmanian populations should not be considered a separate subspecies. A review of *Trichosurus vulpecula* systematics may be warranted.

2.5 Concluding Remarks

- There is limited mtDNA control region variation among *Trichosurus vulpecula* in New Zealand.
- The 60-70 million possums in New Zealand today are descended from a small number of female lineages, perhaps as few as nine female founders.
- There is a lack of structuring to the mtDNA variation found in New Zealand that is best explained by the short time that has passed since a small number of individuals were introduced, and the rapid population growth that has occurred since then.
- Most possums in New Zealand appear to be descended from possums introduced from Tasmania.

Chapter Three

Using mtDNA control region markers to quantify possum movements across buffer zones

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3.1 Introduction

The transmission of bovine tuberculosis (*Mycobacterium bovis*, bovine Tb), from brushtail possums to stock, threatens the New Zealand dairy, beef and deer industries. Tb has been recorded in over 20% of possum populations sampled (Allison 1992). The Animal Health Board's (AHB) objective is to eliminate bovine Tb from New Zealand (Isbister 1991; 1993). To meet this objective, control is undertaken to reduce possum populations and then maintain their densities, on or adjacent to farmland, below the perceived threshold of transmission of Tb. These control, or buffer, zones are designed to minimise the spread of infection, and are based on the disease control models of Barlow (1991a; 1991b; 1993). These models indicated that reduction of possum numbers to less than 25% of pre-control density, followed by maintenance control to hold them at ≤40% of pre-control levels for about 18 years, might eliminate local reservoirs of infection, or prevent them from establishing, so long as immigration of infected possums is not too great (Barlow 2000). Initially, it was thought buffer zones would not only stop the establishment of Tb in areas adjacent to farmland, but also that they would be attractive to possums infected with Tb dispersing from further away, because the reduced possum densities in buffer zones would result in reduced competition for food and nest sites (Cowan and Clout 2000). Theoretically, possums would settle in the buffer zone, rather then disperse across it and into uncontrolled areas. However, in the only study so far conducted on the effect of buffer zones on dispersal, no influence of the buffer zone was detected on either the number of juveniles dispersing, or the average distance dispersed (Cowan and Rhodes 1993). The authors

suggested that the failure to detect any effect on movement due to the buffer zone may have been due to the small number of possums dispersing from the study site, rather than a lack of effect per se (Cowan and Rhodes 1993). Other studies have focused on finding the optimal buffer width for controlling bovine Tb. Three buffer widths were tested: 1-, 3- and 7- kilometres extending into the forest adjacent to a forest/pasture margin. These buffer zones were poisoned once, then recovery of possums (Fraser *et al.* 1998) and changes in the prevalence of bovine Tb (Coleman *et al.* 1998) were measured. In the three years after control, possum density increased by 56% in the 1km buffer, to 35% of pre-control density. Density in the 3 km buffer increased by 28% to 17% of pre-control density, and by 12% in the 7 km buffer to 4% of pre-control density (Fraser *et al.* 1998). Recovery occurred most rapidly in the rear portion of each buffer, adjacent to the uncontrolled forest (Fraser *et al.* 1998). Prevalence of Tb in the possum population was low before control (0.8%) and in the years following control varied from 0% to 1.5% (Coleman *et al.* 1998). It was difficult to determine the effect of the different buffer widths on Tb incidence in cattle, because herds adjacent to the buffer zones had widely varying rates of infection before control. The general trend was of reduction in Tb incidence with all three buffer widths. Taken together, these studies suggest that buffer zones may not limit the movement of dispersing possums per se. This conclusion is tentative at best, because there were few controls in these experiments. Buffer zones will certainly be of benefit because they reduce possum density. This hinders the establishment of Tb, and reduces the number of dispersers, resulting in less risk that any Tb inside the buffer will establish outside the buffer. Wider buffer zones, such as 7- km deep, will be the most effective for providing medium to long-term reduction in the spread of bovine Tb. However, it will also be of use to understand whether buffer zones effect possum movement. One way to achieve this is to measure genetic variation in possum populations on the same, and on opposite sides, of buffer zones, to detect whether there are greater gene frequency differences between populations separated by buffer zones when compared to populations the same distance apart, but on the same side of the buffer.

As discussed in Chapter One (section 1.6), mtDNA can be a useful tool for measuring gene frequency differences. By assaying the genetic variation in possum populations associated with a buffer zone, it may be possible to ascertain whether buffer zones

affect population genetic structure. This will provide a complementary study to that of Cowan and Rhodes (1993) in which direct measures of movement were attempted.

The objectives of the research described in this chapter were as follows:

- Develop a rapid, cost effective and sensitive method of screening the variation in the mtDNA control region of large numbers of possums.
- Use this variation to determine the extent of genetic differentiation in possum populations over small spatial scales.
- If gene frequency differences between populations can be detected, then determine whether poison buffer zones affect this structure, and thus whether buffers affect possum movement.

3.2 Materials and Methods

Recipes for all stock solutions and buffers are provided in Appendix One.

3.2.1 Study populations and DNA extractions

Ear samples were collected from 488 possums from 8 locations associated with two buffer zones in Canterbury (Figure 3.1). The eight locations (designated A – H arbitrarily) were divided into two replicates of four locations each. The replicates (Ashley Gorge and Amuri Range) were separated by 60 km to ensure independence, as this is further than individual possums can generally travel, even when dispersing particularly long distances (Efford 1991). Both buffer zones were established in 1994, when an intensive control operation was undertaken which involved dropping aerial 1080 baits and laying cyanide pellets. Control was repeated in 1998-1999 at both buffer zones. Prior to 1994, both sites had been hotspots for Tb, and as such had been subjected to intermittent control efforts, involving kill-trapping and poison. The habitat in the Ashley Gorge buffer is a mixture of bush cover on the north-west side and farmland on the south-east side. The habitat in the Amuri Range buffer is forest on the north-east side and hill country/scrub on the south-east side.

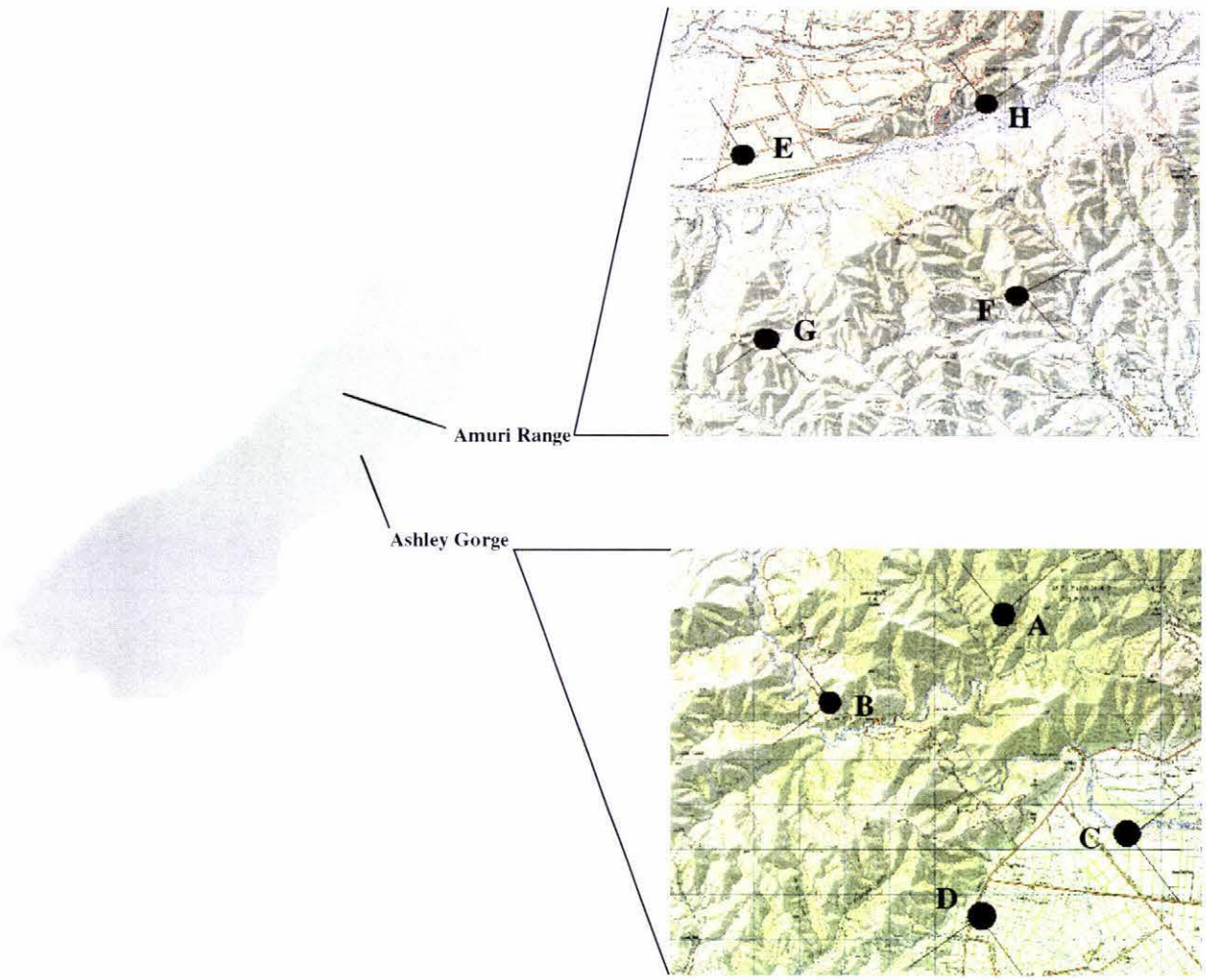

Figure 3.1 Map of the South Island showing the location of the two buffer zones, and the position of the four locations within each buffer zone (black dots). The four Ashley Gorge locations are labelled A – D, and the four Amuri Range locations are labelled E – H. This lettering is arbitrary and used consistently throughout the thesis. The area within which possums were collected at each location is shown (black lines extending from each dot).

Within each replicate, two of the sampling locations were situated on one side of the buffer zone, and the other two sampling locations were directly opposite these, but on the other side of the buffer zone. Locations on the same side were separated by 5 km, and locations directly opposite each other across the buffer zone were 6 km apart. Five km is near the average dispersal distance for possums (Table 1.1). Possums were collected within a 2 km ¼ π radius of the sampling point, extending in a direction away from the other three sampling points within a replicate. Possums were either caught in leg-hold traps spaced 20 – 30 m apart along trap lines, or were killed with cyanide pellets (Ferotox). Possums were collected each day, morphological characteristics recorded (sex, weight, coat colour), and the heads removed and placed on ice. As soon as practicable, ears were removed and stored at -80°C until DNA extraction. Sampling took approximately three weeks for sufficient numbers to be collected (50 – 70 possums per location).

The large number of samples involved required a rapid and cost effective DNA extraction method. A protocol based on that of Zamudio and Sinervo (2000) was developed as follows: 2-3 mm of ear tissue was macerated with a scalpel blade and placed in a tube with 500 µl of 5% Chelex-100 resin (Bio-Rad, CA) and 2 µl of Proteinase K (20 mg/ml). This was incubated at 55°C overnight with constant rotation. In the morning, the sample was refrigerated until required for PCR amplification. DNA extraction using Chelex resin is not only faster and more cost effective than traditional extraction methods using organic solvents, but also lowers the risk of cross-contamination due to the small number of DNA manipulations.

3.2.2 Primer design

New primers were required for use in single stranded conformation analysis (SSCP, see section 3.2.4), as the resolving power of SSCP gels is often lessened for fragments above 300 bp (Hayashi 1991). The 900 bp sequence consensus alignments obtained for 70 individuals around New Zealand were used to map the variation occurring in the possum mtDNA control region. This was achieved by a 'sliding door' approach. For each 10 bp of sequence, the number of variable sites was counted and represented graphically. The results (Figure 3.2) show that although the variation is scattered throughout the segment of control region analysed, there is one main 'hotspot'

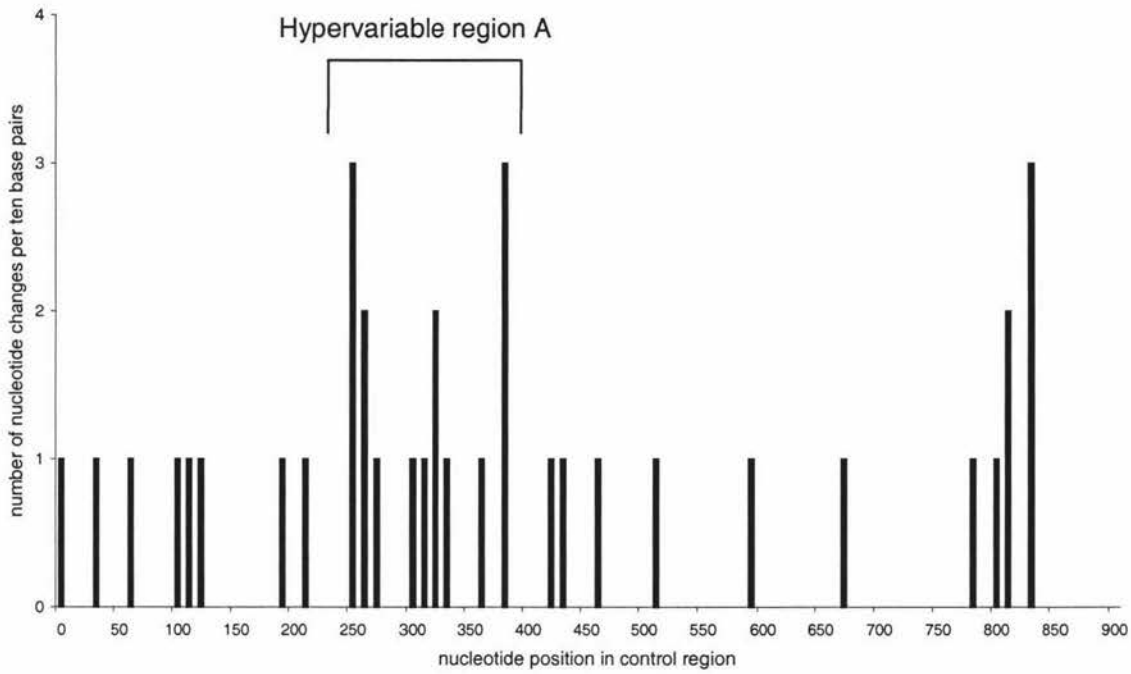

Figure 3.2 Graphical representation of the 900 bp of the control region amplified, showing the relative positions of variable nucleotide sites.

of variability (Hypervariable region A), which occurs in the left variable domain. Hypervariable region A covers 140 bp of the control region and contains 15 (11%) variable sites. Nucleotide sequence analysis of the 900 bp consensus alignment (Table 2.1) revealed that amplifying Hypervariable region A would identify seven of the nine haplotypes (77.8%) identified in section 2.3.2 (Table 3.1). Haplotypes three and nine are identified by nucleotide substitutions occurring outside Hypervariable region A. These two haplotypes are rare, each being represented by one individual only, in the 70 sampled from around New Zealand (Figure 2.9), and are only one nucleotide substitution different from the next most similar haplotype - haplotypes four and eight respectively (Table 3.1).

Two sets of primers that would amplify Hypervariable region A were designed. To maximise the chance of detecting sequence differences using SSCP analysis, primers were designed to minimise the size of the amplified product and were also designed to

Table 3.1 Polymorphic sites in Hypervariable region A of the mtDNA control region of *Trichosurus vulpecula*. Dots indicate that the base is identical to the uppermost consensus sequence, numbering is consistent with that used in Table 2.1. Primers HPTYP1 + HPTYP2 amplify all the variable sites shown, primers SSCP1 + SSCP3 amplify only those shaded grey.

Haplotype	n	109	117	127	179	212	250	254	255	264	268	276	309	311	320	325	336	363	380	386	387	426	433	460	513
Consensus		T	G	A	C	T	T	G	T	T	A	C	A	A	C	A	C	C	T	A	A	C	C	A	T
1	1	C	.	.	T	.	.	A	C	.	.	T	G	.	.	G	.	T	.	G	.	.	.	.	.
2	8	C	.	.	.	.	.	A	C	.	G	.	G	.	.	G	.	.	.	G	.	.	.	G	.
3	1	.	A	G	.	.	C	.	.	.	G	T	.	G	.	.	T	T	C	.	.	T	T	.	C
4	5	.	A	G	.	.	C	.	.	.	G	T	.	G	.	.	T	T	C	.	.	T	T	.	C
5	1	.	.	.	T	.	.	A	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
6	37	.	.	.	T	.	.	A	.	C	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.
7	3	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	3	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
9	1	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.

have high melting temperatures (either through high GC content or through length). The control region is very A + C rich, making characterisation of effective primers difficult. Also, the primers had to be located in a region where there was at least 20 bp of DNA invariant in all possums analysed to date. Four primers: SSCP1 5′- ATACAT-ACTATTCAACATTA-3′, SSCP3 5′- GATGGTGATCTCTCGTGAGG-3′, HPTYP1 5′-CACAGCAAGAGGTAAGTACA -3′, and HPTYP2 5′-TTAGGTACGACCACAG-TTAA -3′ were selected. SSCP1 + SSCP3 amplify 280 bp of the control region, and HPTYP1 + HPTYP2 amplify 475 bp of the control region including the entire 280 bp amplified by SSCP1 + SSCP3 (Figure 3.3). The primer pair SSCP1 + SSCP3 amplify the smallest, most variable portion of the control region possible, for use in SSCP analysis. The primer pair HPTYP1 + HPTYP2 were to be used as sequencing primers, to confirm that bands appearing different on SSCP gels were different in nucleotide sequence, and also as a second set of primers for SSCP analysis, if required. Amplification of the possum mtDNA control region with SSCP1 + SSCP3 and HPTYP1 + HPTYP2, followed by direct sequencing and phylogenetic analysis, confirmed that haplotype 3 grouped with haplotype 4, and haplotype 9 grouped with haplotype 8, as expected.

3.2.3 Amplification of mtDNA control region sequences

Polymerase chain reaction (PCR) amplification of double-stranded DNA was performed in 10 µl volumes, containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals, Germany), and 10-50 ng of template DNA. The typical thermocycling procedure consisted of an initial denaturing step of 94°C for 2 minutes, followed by 25 cycles of 94°C for 20 seconds, 51°C or 62°C for 20 seconds and 72°C for 20 seconds, and a final extension step of 72°C for 4 minutes. For the SSCP primers, the optimal annealing temperature was 51°C, and for the HPTYP primers it was 62°C. All PCR reactions were performed in an iCycler (Bio-Rad, CA).

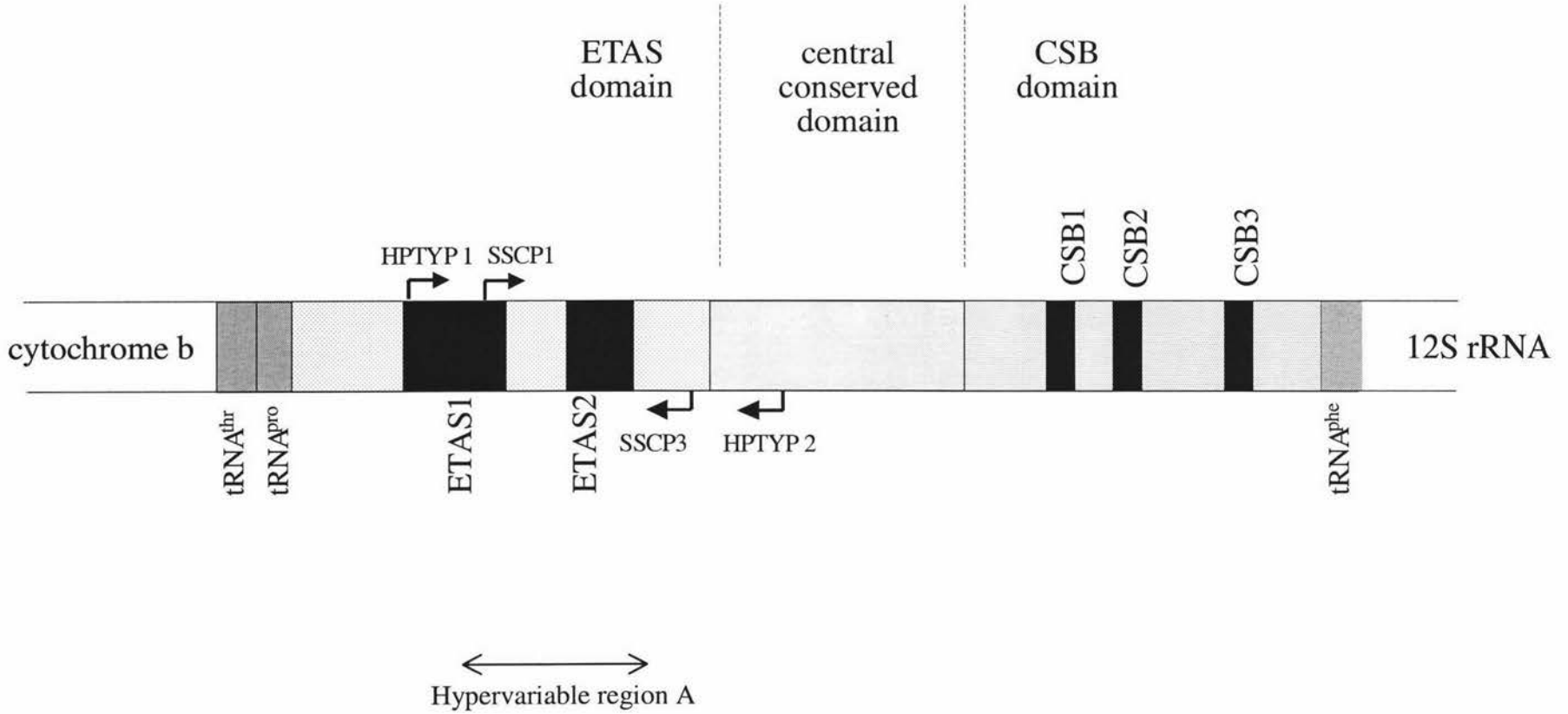

Figure 3.3 Map of the mtDNA control region of possums showing the position of primers SSCP1, SSCP3, HPTYP1 and HPTYP2 (not drawn to scale). Also shown is location of Hypervariable region A. ETAS = extended termination associated sequence (Sbisa *et al.* 1997), CSB = conserved sequence block.

3.2.4 Single-stranded conformation polymorphism (SSCP) analysis

The technique of PCR-SSCP is based on the observation that single-stranded DNA forms specific secondary structures that depend on the precise sequence of bases, and that the rate at which a segment of DNA migrates through a gel is partially dependant on its secondary structure (Orita *et al.* 1989; Friesen *et al.* 1997). Even a single base pair difference between two fragments of DNA can cause them to fold differently, and thus migrate at different rates (Orita *et al.* 1989; Lessa and Applebaum 1993). The analysis of SSCPs can provide a relatively quick and inexpensive way to detect even small numbers of mutations in short fragments of amplified DNA (Lessa and Applebaum 1993), but can be a difficult technique to perfect, as every template has its own set of conditions under which detection of mutation is optimal (Spinardi *et al.* 1991; Lessa and Applebaum 1993).

To develop a rapid screening process for variation in the possum mtDNA control region, several variables known to affect the ability of SSCP gels to detect mutations (Barroso *et al.* 1999; Sunnucks *et al.* 2000, and references therein) were tested: acrylamide concentration (4, 6, 8, 10%), glycerol concentration (0, 2, 4%), buffer concentration (1X and 0.5X), running temperature (4°C vs. room temperature), running time (4, 6, 8, 10,12 hours) and nucleic acid gel stain (EtBr, SYBR Green and SYBR Gold®). The length of the fragment(s) screened was also manipulated by digesting the amplification product with the restriction endonuclease *Bsp*H I. Complete enzyme digestion of fragments was achieved by diluting 30 µl of PCR mix with 20 µl of water and 1X NEB4 buffer, adding 3 units (U) of enzyme to the PCR mix immediately, digesting at 37°C for 1-2 hours, and then adding a further 2 U for digestion at 37°C overnight. Both enzyme digested and undigested PCR amplifications were run on SSCP gels, which allowed the following fragment size combinations to be tested: 200 bp + 80 bp (digested SSCP primer amplification), 280 bp (undigested SSCP primer amplification), 335 bp + 140 bp (digested HPTYP primer amplification) and 475 bp (undigested HPTYP primer amplification). The optimal set of running conditions was established through a series of trials, and the final protocol adopted for routine analysis, as described below.

Five microlitres of unpurified amplified DNA was mixed with 5 µl of denaturing loading buffer (deionised formamide and 0.01% xylene cyanol), boiled for 7 minutes,

immediately cooled on ice, and then loaded onto a non-denaturing 8% polyacrylamide gel (37.5:1 acrylamide:methylbisacrylamide), containing 0.5X TBE, 4% glycerol, 250 µl ammonium persulphate (0.1 g/1ml) and 30 µl TEMED. Gels were pre-chilled to 4°C. A V-16-2 (Life Technologies, MD) double gel rig (170 x 150 x 1.5 mm) was used so that two gels, and thus 40 samples could be run simultaneously. Eighteen possum samples were loaded onto each gel and a size standard (1 kb+ ladder, Life Technologies, MD) was run at either end of the gel. The running buffer was 0.5X TBE, which was also pre-chilled to 4°C. The gels were run at 4°C, at constant power (7 W) for 12 hours, stained in SYBR® Gold nucleic acid gel stain (Molecular Probes, OR) for one hour, visualised via UV trans-illumination, and recorded via a gel documentation system (Insta Doc™ System, Bio-Rad, CA).

3.2.5 Confirmation of haplotypes detected by SSCP

Running a ladder at either side of the gel provided a means to check that the gel had run as expected, and allowed an initial assessment of whether SSCP bands on different gels represented the same haplotype. Differences in band mobility detected by SSCP analysis were scored as putative haplotypes. Direct sequencing of at least three individuals (where available) with the haplotype was used to confirm sequence differences. When the SSCP analysis was performed with the SSCP primers, either HPTYP1 or HPTYP2 was used as the sequencing primer. When the SSCP analysis was conducted with the HPTYP primers, either Tv5'F or Tv5'R was used as the sequencing primer. Sequencing was carried out as described in section 2.2.5, except that sequencing reactions were completed in an iCycler (Bio-Rad, CA) with the following thermocycling profile: an initial denaturing step of 94°C for 30 seconds, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

3.2.6 Analysis

OLIGO 4.0-s (National Biosciences, Inc) and Amplify 1.2 (University of Wisconsin, WI) were used as aids to design the primers. DNA sequences were aligned and edited using Sequencher™ 3.1.1 (Genecodes, Corp). Potential restriction sites were found using Sequencher 3.1.1 and Gene Tool Lite 1.0 (BioTools, Inc). Phylogenetic analyses were made using Paup* 4.0b4a (Swofford 2000). ϕ_{ST}, an analogue of Wright's F_{ST} that incorporates information on sequence divergence among haplotypes, was calculated

following the methods of Excoffier *et al.* (1992) using Arlequin 2.0 (Schneider *et al.* 2000).

3.3 Results

3.3.1 Detection of haplotypes by PCR-SSCP - Pilot Study

Initial SSCP trials were aimed at determining the optimum length of the fragment to be screened. Several previous studies had indicated that fragment length is extremely important in determining the sensitivity of SSCP analysis (Orita *et al.* 1989; Sheffield *et al.* 1993; Girman 1996; Barroso *et al.* 1999). Hayashi (1991) suggested that the probability of detecting a one-base substitution by mobility shift in at least one of the DNA strands is ~99% for fragments 100 - 300 bp and ~89% for fragments 300 - 450 bp. Conversely, Ortí *et al.* (1997) were able to detect all sequence differences in a 775 bp long fragment, and Friesen *et al.* (1997) found no difference in SSCP sensitivity with fragments ranging in size from 100 - 500 bp. The 280 bp that the SSCP primers amplify falls within the range where one can expect 99% mutation detection of single base pair changes, and can be further cleaved into two fragments of 200 bp and 80 bp using the restriction enzyme *Bsp*H I. The HPTYP primers amplify 475 bp of mtDNA control region, so the probability of detecting all single base pair mutations using this fragment is presumably less than 89%, based on the results of Hayashi (1991). When digested with the restriction enzyme *Bsp*H I, this fragment is cleaved into two fragments of 335 bp and 140 bp.

All four types of fragment(s) possible, (SSCP and HPTYP primers, digested and undigested) were trialed on SSCP gels under optimised conditions, using individuals with each of the seven haplotypes theoretically detectable (based on sequence differences within the fragments).

Three factors that were important for producing interpretable results were identified when optimising the SSCP running conditions. First, the method was very sensitive to small changes in temperature across the gel. At first, gels run at 4°C for more than a few hours tended to have notable warping of bands across the gel (Figure 3.4a). This

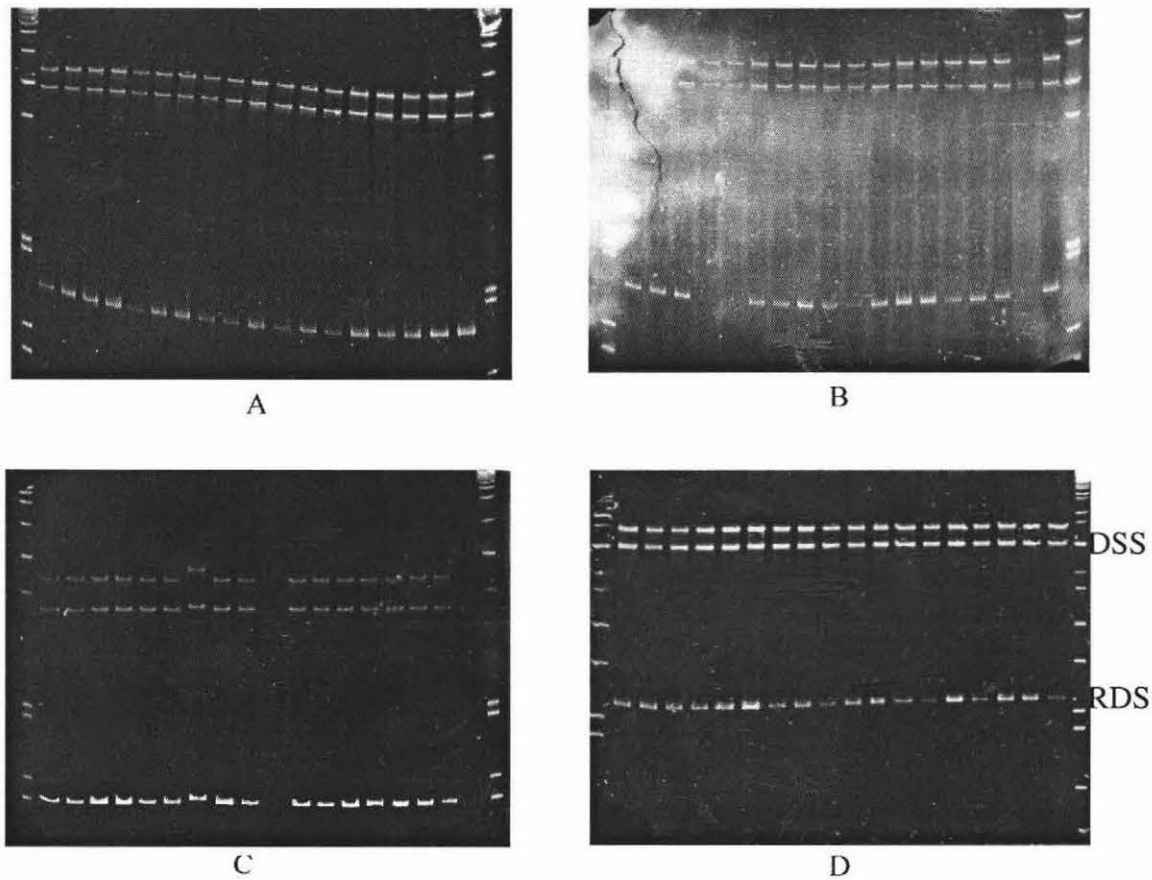

Figure 3.4 Gel picture showing several factors that affect mutation detection ability: (A) uneven surface temperature causes warping, (B) handling with nitrile gloves causes smearing at the edges, (C) staining in ethidium bromide overestimates the ratio of RDS:DSS. Also shown is a gel run once the above factors had been negated (D). It shows that among 18 males from location A, only one haplotype is present. DSS = denatured single stranded DNA, RDS = renatured double stranded DNA.

was eventually eliminated by reorienting the gel rig so that gels faced the cold room fan, rather than being perpendicular to it. When not in this position, the uneven flow of cool air caused a temperature gradient across the gel. Second, handling the acrylamide gels with nitrile gloves before and after they were stained caused significant smearing across the gel, making interpretation very difficult (Figure 3.4b). This smearing was produced whether the gel was stained in ethidium bromide, SYBR green or SYBR gold, suggesting that the smearing was caused by the nitrile gloves reacting with the acrylamide gel, rather than with the nucleic acid stain. Handling gels with latex gloves resolved this problem. Third, ethidium bromide staining tended to over-estimate the ratio of renatured double-stranded DNA to single-stranded DNA, because it does not stain single-stranded DNA very effectively (Figure 3.4c). To negate this, SYBR gold was used because it has a higher affinity for single-stranded DNA. Once the above factors had been resolved, gels were clear and easy to score (Figure 3.4d). The only fragment that was able to detect all seven haplotypes was the largest: the 480 bp fragment amplified by the HPTYP primers (Figure3.5). Because this was the largest fragment, it also took the longest time to satisfactorily separate the two bands (Table 3.2). The HPTYP primers were chosen for use in the buffer zone analysis.

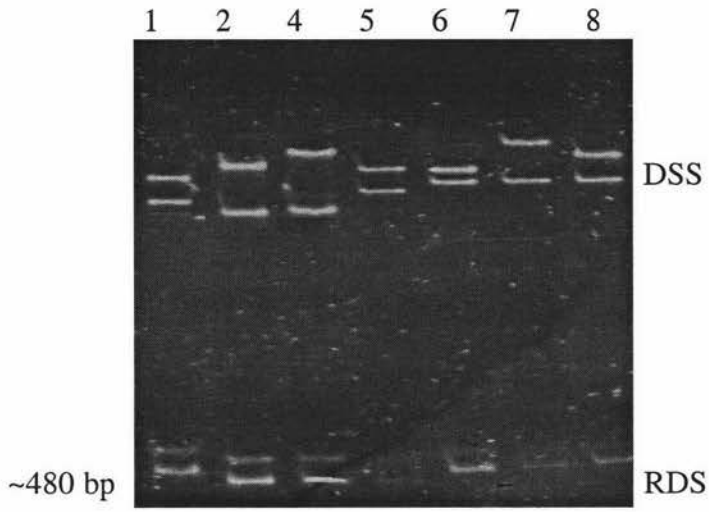

Figure 3.5 SSCP gel of possum mtDNA amplified by the HPTYP primers, showing that all seven haplotypes are clearly differentiated by mobility shifts in the DNA bands. Both denatured single stranded (DSS) and renatured double stranded (RDS) DNA are detected. The RDS runs at approximately 480 bp. Haplotypes are numbered as described in Chapter Two

Table 3.2 Number of haplotypes detected, and time taken to run on an SSCP gel, for various amplifications of possum mtDNA.

Fragment	Number of haplotypes detected	Time needed to separate fragments (hours)
SSCP primers; digested with *Bsp*H I	6	6
SSCP primers; undigested	5	9
HPTYP primers; digested with *Bsp*H I	6	10
HPTYP primers; undigested	7	12

3.3.2 Detection of Haplotypes by PCR-SSCP - Buffer Zone Study

For each of the eight locations (Figure 3.1), a random sample of 9-18 males and 9-18 females were chosen for an extensive analysis of mtDNA control region variation associated with poison buffer zones (Table 3.3). A total of 13 SSCP gels were required to run all 234 samples analysed. Initial SSCP analysis suggested that there were only two variants, one of which was very common, while the other occurred only in three individuals (two males and one female), all from location H. Comparison gels were run, which confirmed the homology of the common variant from different gels. Identical sequences were confirmed by sequencing a selection of eight individuals (one male and one female from each of the eight locations) exhibiting the common variant, and all three individuals with the rare variant. Direct sequencing established that there were no nucleotide changes among individuals that appeared, from analysis of the SSCPs, to possess the same haplotype. Both the variants detected were haplotypes that had previously been identified (section 2.3.1). The common haplotype was haplotype 6 (also the most common haplotype found in New Zealand as a whole), and the rare haplotype was haplotype 1. Haplotype 6 was the only haplotype found by sequencing six individuals chosen at random from each of the two buffer zone sites for inclusion in the survey of variation around New Zealand (section 2.3.1). Haplotypes 1 and 6 are the two haplotypes that were detected in Banks Peninsula, which is geographically the closest site to the location of the buffer zones in this analysis.

Of the 234 individuals analysed, 231 (98.7%) had haplotype 6, and three (1.3%) had haplotype 1. All possums from the four localities in Ashley Gorge had haplotype 6, as did all possums from three of the four localities (E, F, G) in Amuri Range. At the fourth locality (site H), 83.3% had haplotype 6 and 16.7% had haplotype 1. The overall ϕ_{ST} (= 0.074, P = 0.003) was low, indicating that only 7.4% of the variation was due to differences between populations and hence there is very little population differentiation, even between sites more than 60 km apart. Table 3.4 shows the nucleotide differences between populations (expressed as ϕ_{ST} values) and whether or not each result is significantly different from random. All of the eight locations had possums of both coat colours, and in most sites there were roughly equal proportions of both colour morphs (Table 3.3), suggesting there was no correlation between coat colour and haplotype. A chi-square test confirmed that coat colour was not associated with haplotype in the

buffer zones ($\chi^2 = 11.73, p = 0.700$). A test of the association between coat colour and haplotype, encompassing all individuals analysed (that is, 70 from chapter two and 234 from chapter three) found that there was not a significant interaction between coat colour and haplotype ($\chi^2 = 16.53, p = 0.487$).

Table 3.3 Number of individuals and haplotypes sampled at each of the eight locations in the buffer zones study. Locations A-D comprise the Ashley Gorge replicate, locations E-H comprise the Amuri Range replicate. Also shown is the percentage of black possums sampled at each location. The percentage of grey possums sampled at each site is equal to 1 - (% black).

	Number of individuals assayed				
Site	Male	Female	Total	Number of haplotypes	% black possums
A	18	18	36	1	41.6
B	18	18	36	1	55.6
C	18	18	36	1	44.4
D	18	18	36	1	22.2
E	18	9	27	1	59.2
F	18	9	27	1	70.4
G	9	9	18	1	38.9
H	9	9	18	2	44.4
				Average	46.1

Table 3.4 Pairwise comparisons of the ϕ_{ST} values among groups of brushtail possums associated with two buffer zones. The lower diagonal shows the ϕ_{ST} values, a measure of nucleotide difference between populations. The upper diagonal gives the probabilities of the observed ϕ_{ST} value occurring by chance in 1,000 permutations. None of the comparisons are significant at the 0.05 level. Labels A – H refer to locations A – H, as shown in Figure 3.1.

	A	B	C	D	E	F	G	H
A	-	0.991	0.991	0.991	0.991	0.991	0.991	0.117
B	0.000	-	0.991	0.991	0.991	0.991	0.991	0.117
C	0.000	0.000	-	0.991	0.991	0.991	0.991	0.126
D	0.000	0.000	0.000	-	0.991	0.991	0.991	0.135
E	0.000	0.000	0.000	0.000	-	0.991	0.991	0.180
F	0.000	0.000	0.000	0.000	0.000	-	0.991	0.162
G	0.000	0.000	0.000	0.000	0.000	0.000	-	0.108
H	0.068	0.066	0.046	0.046	0.076	0.080	0.080	-

3.4 Discussion

3.4.1 Development of SSCP technique

SSCP is a time-consuming technique to implement, because of the number of parameters that can be varied to maximise mutation detection. Over a month was required to fully optimise conditions in the present study, so that maximum sensitivity and reproducibility were achieved. However, once the technique had been fully optimised, it allowed rapid and sensitive mutation detection. The 234 individuals were processed in less than ten days, and gels were always clear, consistent and easy to interpret.

Unexpectedly, it was the largest fragment, rather than the smallest, that was best able to discriminate among the haplotypes. It has been previously suggested (Sheffield *et al.* 1993; Barroso *et al.* 1999) that it is not only the size of the fragment that is important, but also the location of the sequence differences within the fragment, that plays a key

role in the discrimination power of SSCP analysis. Mutations towards the end of a fragment may be more difficult to detect (Barroso *et al.* 1999). This was not the case in the analysis conducted here. Haplotypes that were impossible to differentiate, via SSCP, with the SSCP primers were characterised by 8 - 9 nucleotide substitutions, scattered throughout the fragment. Notably, the SSCP primers were able to differentiate between haplotypes 7 and 8, which are only one base pair different in this amplicon, but not between haplotypes 4 and 7, which are ten base pairs different. Furthermore, the single base pair difference between haplotypes 7 and 8 is reasonably close to the end of the amplicon. Similarly, Ostellari *et al.* (1996) could detect no variation in mobility of a 229 bp fragment among 32 individuals, but sequencing revealed seven mutations, together defining seven haplotypes. Clearly the sensitivity of SSCP analysis is critically dependent on the nature of the fragment subjected to the analysis. These results illustrate the importance of direct sequencing, not only of individuals with different SSCP patterns to confirm they are different in sequence, but also of individuals that appear identical by SSCP analysis, to confirm that they have identical sequences. Given that some amplicons fail to distinguish all sequence differences (false negatives), the chance of a type II error (failure of SSCP to detect a difference that occurs in the sequence) is increased. Possibly the best way to reduce the potential for false negatives when using SSCP analyses in population genetic surveys is to sequence at least one representative of each haplotype from each of the populations where it occurs (Shaffer *et al.* 2000). Despite the above limitations, SSCP is an excellent technique for screening large numbers of individuals rapidly and cost-effectively. The primers developed in the present study are able to characterise most of the variation detected in New Zealand and no false positives or negatives were detected.

3.4.2 Inferring structure and movement patterns from mtDNA variation

There was only one common variant (haplotype 6) among all eight locations in the buffer zones. These localities were separated by as much as 60 km. Furthermore, this variant was the only one found at a nearby location (Banks Peninsula, section 2.3.1) suggesting that there is a lack of variation, even over wide spatial scales, in the Canterbury region. The very low levels of variation found associated with these buffer zone meant that it was not possible to infer genetic structure on a small spatial scale using mtDNA. The low levels of variation are not unexpected, for two reasons. First,

the buffer zones are in the South Island. The previous analysis of variation around New Zealand (section 2.3.1) showed that there may be as few as three haplotypes in the South Island. Second, the possums are associated with buffer zones that have been periodically poisoned. Every time possum numbers are reduced by control, there is potential for a decrease in genetic variability. The sudden reduction in numbers is analogous to a genetic bottleneck. Numbers recover fairly quickly, so the bottleneck does not last long, but the circumstances are repeated at regular intervals. The sampling locations in this survey were designed to be outside the area targeted for poisoning, however the poison was aerally sown, so some drift could be expected to have occurred. Furthermore, the buffer zone areas were subjected to more widespread control prior to 1994. The extent to which regular poisoning of an area will reduce variability depends on how long poisoning has been occurring, the extent to which numbers are reduced, and the way in which possums move following such control operations. At least initially, most recovery is likely to be from immigration. The effect on genetic variability will depend on the size of the area controlled relative to the surrounding uncontrolled areas that provide immigrants. If survivors from the immediate vicinity increase their home ranges to incorporate the newly vacated area (the 'vacuum effect', Efford *et al.* 2000), then it is likely that variability will be lower than if possums from further away (and thus from a wider pool of potential variation) colonise the area.

The common haplotype found in the buffer zone analysis (haplotype 6) occurred in 98.7% (231) of the possums surveyed. Of the 231 possums with haplotype 6, 111 (48.1%) had black fur and 120 (51.9%) had grey fur. This haplotype is suspected to have originated as a Tasmanian lineage (section 2.3.2). Possums imported from Tasmania are thought to have been predominantly black individuals, as black fur was in demand by the fur trade (Pracy 1974). Even allowing for some bias due to differences in judgement by different collectors, it is clear that there is no systematic bias for haplotype 6 to be represented by either coat colour, as confirmed by the chi-square test, which showed no correlation between these two variables. All of the three individuals possessing haplotype 1 (potentially a Victorian or New South Wales lineage), were grey, as were all nine individuals possessing haplotypes 1 and 2 in the earlier New Zealand wide survey (section 2.3.1), which group to form clade b. In general, chi-square tests failed to detect any association between coat colour and haplotype, however the small numbers of individuals with some haplotypes may have masked any

association. More extensive sampling would allow assessment of whether there is any correlation between coat colour and haplotype and sampling from Australia should allow identification of the maternal origin of clade b. Because clades a and c are clearly mixed for coat colour, at this stage it seems unlikely that fur colour reflects the maternal origin of brushtail possums in New Zealand.

Although it was not possible to detect population genetic structure on a fine scale in these South Island buffer zones, SSCP analysis is likely to be of use in areas with higher levels of variation. The survey described in Chapter Two indicated that the North Island has greater haplotypic diversity than the South Island, and that certain parts of the North Island have particularly high variability. Australia is also likely to have much higher levels of variation than those found in the South Island, based on the limited analysis of Australian possums detailed in Chapter Two, and the fact that Australian populations are unlikely to have undergone recent, extensive genetic bottlenecks. In areas of higher variability, it may be possible to detect structure over small spatial scales, provided there are at least two reasonably common haplotypes. With the genetic markers identified and the SSCP technique developed here for brushtail possums, it would be a simple task to conduct a pilot survey of an area of interest and assess overall levels of variation, before the expense of an extensive survey. Areas that show two or more common haplotypes may justify further intensive analysis.

The study of genetic structure across a buffer zone described here was conducted as one part of a larger study utilising two types of markers: the maternally inherited mtDNA control region markers described in this chapter, and a set of bi-parentally inherited nuclear microsatellite markers. Although beyond the scope of this thesis, these microsatellite markers detected higher levels of variation than the mtDNA markers, and hence may be more applicable to studies of genetic variation over small spatial scales, particularly in the South Island.

3.5 Concluding Remarks

- SSCP analysis is an effective method for screening mtDNA control region variation in large numbers of individuals rapidly and cost-effectively.
- There was no correlation between fragment size and sensitivity for mutation detection in SSCP gels, as has been widely reported.
- Levels of variation in South Island buffer zones were extremely low, such that it was not possible to detect genetic differentiation over small spatial scales, and hence determine whether poison buffer zones affect genetic structure of possum metapopulations.

Chapter Four

Discussion and Conclusions

4.1 Synthesis

Direct sequencing of 900 bp of the mtDNA control region of 70 brushtail possums revealed nine haplotypes, and an overall paucity of genetic variation amongst New Zealand possum populations. All 60 million brushtail possums appear to trace their ancestry back to as few as nine female founding lineages. Further, there is an apparent lack of correlation between haplotype and phenotype, for both geographic location and coat colour of individuals. Variation is not distributed evenly within New Zealand. The most common haplotype was found in 67% of possums sampled, and 6 haplotypes (66.7%) were present in 1-3 possums only. The South Island, Stewart Island and Chatham Island have much lower levels of variation than the North Island. Even within the North Island, populations vary from having no haplotypic diversity (such as the Auckland and Wellington regions) to comparatively high haplotypic diversity (such as Cape Palliser and the Tararua Ranges). Of the nine haplotypes found, five appear to have Tasmanian origins, including the most common haplotype (6).

The variation associated with poison buffer zones in the South Island was also surveyed. A total of 234 individuals were assayed, and 98.7% of these possessed haplotype 6, with the remaining 1.3 % having haplotype 1. The dearth of variation found within the buffer zone meant that it was not possible to determine whether the buffer zone had an effect on population genetic structure. The low levels of variation in the buffer zones are likely to be a reflection of low overall levels of variation in the South Island, resulting from the initial bottleneck in combination with repeated bottlenecks caused by repeated poisoning of the buffer, rather than a reflection of high levels of gene flow between locations. Again, there was no clear correlation between coat colour and haplotype suggesting that it is not possible to use coat colour as a rough

indicator of maternal lineage, in contrast to the allozyme results of Triggs and Green (1989). Taken together, these results suggest low overall levels of variation, and lack of genetic structure.

4.2 Indirect methods for estimating possum movement

The small numbers of individuals from each site included in the survey of mtDNA control region variation around New Zealand (Chapter Two) meant that it was not possible to use these data to measure gene frequency differences between sites, and infer gene flow using populations genetics models. Sampling was conducted on a scale that would enable detection of population structure should it exist. Locations within a replicate were separated by 5 – 6 km, which is within the known dispersal capabilities of brushtail possum (Table 1.1). The two replicates were separated by 60 km, which is beyond the known dispersal capability of possums. The longest possum dispersal event ever recorded by direct methods was 41 km (Efford 1991). If structure had been detected within a replicate, this would have suggested that genetic exchange was not occurring over these small distances, and it would have been possible to make inferences about whether the buffer zone was contributing to this lack of exchange. If structure had been detected between replicates, but not within replicates, this would imply that possums readily exchange individuals between populations separated by 6 km or less, but not between populations separated by 60 km or more. Again, inferences about the effect of buffer zones on genetic exchange would have been possible. Unfortunately, evaluation of genetic exchange, or the effect of buffer zones, was not possible because of the extreme lack of variation. One way to interpret the results is that there is high gene flow between all localities, and thus there are no gene frequency differences between them. As discussed in section 1.3.3.4, direct methods may be biased towards recording shorter dispersal events, yet it seems highly unlikely that dispersal estimates are, on average, incorrect by an order of magnitude, especially when one considers the number of studies conducted on possum dispersal in New Zealand, (in the last ten years more than 12 papers dealing directly with possum dispersal have been published in refereed journals). A more plausible explanation is found when one considers that the South Island as a whole has a lack of variation compared with the

North Island, and when one considers the likely genetic consequences of a buffer zone regime - poisoning at regular intervals followed by immigration of surrounding possum populations.

As a consequence of the lack of variation in the buffer zones, and consequent inability to apply population genetics models, it has not been possible to evaluate whether mitochondrial markers are able to detect the effect of potential barriers to movement, such as buffer zones. It certainly seems unlikely that this method using a mitochondrial marker, will be of use in the South Island, or in other areas of low variability, such as offshore islands and the Auckland region. Other places seem much more variable, such as the Tararua Ranges and Cape Palliser, and so the method may be of use in these areas. It is unclear whether the lack of variation in the South Island buffer zones was due to their location, the effects of repeated poisoning, or high levels of movement unaffected by the buffer zone (or a combination of these factors), thus the method may or may not be of utility in determining the effect of barriers on possum movement. It would be of great value to know whether poison buffers are effective in altering the movement behaviour of possums, as buffers are one of the most commonly used methods to attempt the halt of Tb spread. In this situation, direct methods are likely to always have the problem of small sample sizes (as was the case with the study of Cowan and Rhodes 1993), because poisoning reduces possum density to low levels, leaving few possums to disperse. Thus, it would be well worth attempting a similar analysis to the one described here with a North Island buffer zone.

This study of mtDNA variation in brushtail possums in New Zealand was designed to determine whether there will be sufficient levels of variation to measure gene flow, by applying population genetics models such as Wright's F_{ST} calculation (Wright 1951, 1953). One important aspect to applying indirect methods to measures of gene flow is the question of whether or not possum populations in New Zealand will be at equilibrium. The best way to determine this will be to compare estimates obtained with direct and indirect methods to determine whether they are somewhat in agreement. Several different measures of population differentiation, such as microsatellites and mitochondrial markers, should be used for indirect methods. Considering the history of possums in New Zealand, it seems likely that populations will not be at equilibrium, and that artefacts of the colonisation history will still be apparent in possums in New

Zealand today. The main reasons for this are that first; there has been repeated colonisation of uninhabited parts of New Zealand for the last one hundred and fifty years. Possums have only been recorded in the Northland and Fiordland areas in the last fifteen years (Clout and Ericksen 2000). Colonisation will have resulted in continual founding of new populations by few individuals, so founder effects will not just have occurred during the initial introduction, but are likely to have been occurring within the last fifteen years, and may still be occurring today (Le Corre and Kremer 1998). Second, *Trichosurus vulpecula* has been an extraordinarily successful coloniser of New Zealand, such that the population has exploded from roughly 300 individuals 150 years ago (Pracy 1974), to a likely population today of over 65 million (Montague 2000). When populations expand in numbers very rapidly, stochastic lineage extinction is markedly slowed (Avise *et al.* 1984; Boileau *et al.* 1992). Thus, it takes much longer for gene frequency differences to accrue between populations. For these reasons, the application of indirect assessments of movement in *Trichosurus vulpecula* in New Zealand will need to be approached with caution.

4.3 SSCP as a method for surveying large numbers of individuals

In order to detect small variations in gene frequencies among populations, large numbers of individuals will usually need to be sampled. Direct DNA sequencing of individuals is generally the best method in such studies, as every mutational change is detected. However, it is time consuming and expensive to conduct a sequencing project involving numerous individuals. Other methods such as restriction fragment length polymorphisms (RFLPs) will only assay a portion of the total variation, but are much quicker and technically easier to implement. SSCP is a method that has the benefits of direct sequencing (detection of fine-scale variation, such as a single substitution in several hundred base pairs), without the time and expense associated with DNA sequencing. Optimisation can be time-consuming, but once completed, large numbers of individuals can be processed in a short time period. Sequencing is required only for each haplotype, rather than each individual. One perceived drawback to the use of SSCP is that only small segments of DNA can be reliably processed to ensure complete mutation detection. The results of Hayashi (1991) are often cited as evidence that

fragments shorter than 300 bp (or enzyme digested to fall below this threshold) should be used for SSCP to increase the chances of complete mutation detection. However, there is increasing evidence that often there is not a direct correlation between fragment length and sensitivity of mutation detection (Sheffield *et al.* 1993, Ostellari *et al.* 1996, this study). Clearly, there is an interaction between fragment length and fragment composition, such that type and position of nucleotide changes in the fragments are as important as the length of the fragment in determining whether or not there will be a change in conformation. This was evidences by the accurate detection of a single base pair difference between two fragments amplified by the SSCP primers, but the failure of these primers to detect the difference between two fragments differing by 10 bp (section 3.4.1). The widely reported direct correlation between fragment length and detection sensitivity is misleading and should be reviewed. A better approach is to conduct an initial survey of the sequence variation in the portion of genome of interest, to identify the most variable regions. These will be the best candidates as effective SSCP markers, and pilot surveys will determine the most effective primers for maximum mutation detection. Length should be minimised where possible, but not to the extent that highly variable portions of the genome are excluded.

4.4 Future Directions

There are several future research directions this work could take. The first would be a more extensive survey of those regions identified as having high variability (section 2.3.1) to identify whether there are further mtDNA control region haplotypes not yet uncovered. This is of interest because it will allow a more accurate assessment of the number of female lineages that New Zealand brushtail possums are descended from. The second direction is to conduct a more extensive survey of key areas in Australia, especially Victoria and New South Wales, to help resolve whether the three clades apparent in phylogenetic analyses of New Zealand possums represent three maternal origins of possums in New Zealand. This may have a bearing on the way in which possum control is implemented in New Zealand The third direction is to use the SSCP system developed in this thesis to analyse the variation associated with North Island buffer zones, in an attempt to resolve the question of whether buffer zones affect possum movement based on changes in gene frequencies in populations on either side

of a buffer compared to populations on the same side. Now that the system has been developed, a rapid and sensitive survey of mtDNA control region variation is readily available, and its implementation should be a simple matter, both in New Zealand and Australia. The fourth direction that this work can take is the analysis of microsatellite variation in the same buffer zone possums analysed for mtDNA variation. That work is currently being undertaken (Sarre *et al* in prep). The application of two different markers (one maternally inherited, the other bi-parentally inherited) to analyse gene frequency differences between populations was initiated because it would allow the possibility of detecting sex-biased differences, if the buffer zone affected one sex differentially over the other sex. Unfortunately this will not be possible because of the lack of mitochondrial variation detected. The fifth direction this work could take is to apply the general techniques to other introduced species in New Zealand to address fundamental issues of population genetics of pest species in this country.

Appendix One

Recipes for buffers and stock solutions

Lysis Buffer

10 mM NH_4HCO_3

0.14 mM NH_4Cl

Resuspension Buffer

0.05 M Tris-HCl pH 7.5

0.1 M NaCl

0.001 M EDTA

SET buffer

100 mM Tris-HCl

100 mM NaCl

1mM EDTA

TBE (for agarose mini-gels)

134 mM Tris

75 mM Boric Acid

2.5 mM EDTA

TBE (for acrylamide SSCP gels)

89 mM Tris

89 mM Boric Acid

2.5 mM EDTA

Appendix Two

DNA extraction protocols

Extraction of DNA from Tissue (frozen, stored in ethanol or fresh)

1. Macerate the tissue as finely as possible with a sterile scalpel, on a sterile surface.
2. Transfer the tissue to a 1.5 ml eppendorf tube with 400 ml SET buffer, 50 µl Proteinase-K, and 50 µl SDS.
3. Incubate overnight rotating at 45 - 65°C (ideally 55°C).
4. If tissue stored in ethanol add 50 µl 5M NaCl, otherwise proceed to next step.
5. Add an equal volume (500 µl) phenol. Rock for 30 minutes and centrifuge for 5 minutes at 10,000 rpm. Two layers will form: DNA at the top and phenol at the bottom.
6. Remove the top DNA layer to a fresh 1.5 ml eppendorf tube. Discard the tube containing the phenol in a phenol waste container as appropriate.
7. If the DNA layer is still rather 'cloudy' then repeat the phenol step, otherwise proceed to next step.
8. Add 500 µl phenol: chloroform: isoamyl alcohol (25:24:1). Rock for 30 minutes, centrifuge at 10,000 rpm for 5 minutes, then remove and discard the bottom layer.
9. Repeat step 8.
10. Add 500 µl chloroform: isoamyl alcohol (24:1). Rock for 30 minutes, centrifuge at 10,000 rpm for 5 minutes, then remove and discard the bottom layer.
11. Repeat step 10 if high quality DNA is required.
12. Centrifuge samples again for 2 minutes and remove all remaining chloroform.
13. Precipitate the DNA by adding 60 µl 3M NaOAc pH 5.2, and filling the tube with ice-cold 100% ethanol (about 1 ml is required). Rock for 30 minutes.
14. Remove DNA spools with a P1000 tip and place in 1 ml room temperature 70% ethanol. Spin 30 minutes at 13,000 rpm. If DNA has not spooled, then spin tubes for

30 minutes at 13,000 rpm, and wash twice with 70% ethanol, instead of once, as explained in step 15.

15. Remove supernatant and add 1 ml 70% ethanol. Spin 30 minutes at 13,000 rpm.
16. Remove all traces of ethanol and dry upside down on tissue paper.
17. Resuspend in sterile H_2O.

Extraction of DNA from blood

1. Extraction of white blood cells

(The number of samples that can be processed at one time is limited by the capacity of the centrifuge - 8 for the Sorvall RC 28S)

1. Label 8 125 ml flasks and 8 polypropylene centrifuge tubes with appropriate details.
2. Place flasks on ice and add 15 ml cold lysis solution to each. Add ~5 ml of blood (if there is less blood add more lysis solution so that the final volume is 20 ml).
3. Mix blood in vacutainer gently with a transfer pipette, then suck up blood and transfer to flask.
4. Rinse out vacutainer with 3 ml cold lysis solution and use transfer pipette to add to flask. Swirl vigorously.
5. Leave on ice for 30 minutes.
6. Pour solution into centrifuge tubes, and rinse out flasks with 3 ml of cold lysis solution.
7. Balance tubes carefully, adding more lysis solution if necessary. Spin for 15 minutes at 7000 rpm at 4°C.
8. Decant and discard supernatant.
9. Add 2 ml of cold lysis solution to the centrifuge tube on the opposite side to the pellet. Gently agitate the tube so that the surface of the pellet is washed with the lysis solution, in order to remove unwanted matter, e.g. red blood cells, from the pellet and the sides of the tube.
10. If necessary, repeat once or twice by adding fresh lysis solution until the pellet is reasonably clean. It will still be quite pink, but the cleaner the better.

11. Wipe inside of tube with a tissue to remove any remaining matter from the walls, being careful not to disturb the pellet. Air dry upside down on tissue paper.
13. Resuspend the in 450 µl resuspension buffer and vortex vigorously.
14. Transfer to 1.5 ml tubes and freeze if necessary until required.

2. Extraction of DNA from white blood cells

1. If frozen, thaw samples. Add 40 µl 10% SDS and 20 µl Proteinase-K (20 mg/ml).
2. Incubate overnight rotating at 45 - 65°C (ideally 55°C).
3. Add an equal volume of phenol (500 µl). Rock for 30 minutes and centrifuge for 5 minutes at 10,000 rpm. Two layers will form: DNA at the top and phenol at the bottom.
4. Remove the top DNA layer to a fresh 1.5 ml eppendorf tube. Discard the tube containing the phenol in a phenol waste container as appropriate.
5. Add 500 µl phenol: chloroform: isoamyl alcohol (25:24:1). Rock for 30 minutes, centrifuge at 10,000 rpm for 5 minutes, then remove and discard the bottom layer.
6. Repeat step 8.
7. Add 500 µl chloroform: isoamyl alcohol (24:1). Rock for 30 minutes, centrifuge at 10,000 rpm for 5 minutes, then remove and discard the bottom layer.
8. Repeat.
9. Centrifuge samples again for 2 minutes and remove all remaining chloroform.
10. Precipitate the DNA by adding 50 µl 3M NaOAc pH 5.2, and filling the tube with ice-cold 100% ethanol (about 1 ml is required). Rock for 30 minutes.
11. Remove DNA spools with a P1000 tip and place in 1 ml room temperature 70% ethanol. Spin 30 minutes at 13,000 rpm. If DNA has not spooled, then spin tubes for 30 minutes at 13,000 rpm, and wash twice with 70% ethanol, instead of once, as explained in step 15.
12. Remove supernatant and add 1 ml 70% ethanol. Spin 30 minutes at 13,000 rpm.
13. Remove all traces of ethanol and dry upside down on tissue paper.
14. Resuspend in sterile H_2O.

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