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.

Joanne R. Chapman
Molecular Ecology, Institute of Molecular BioSciences,
Massey University

2001

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.

Fumagalli, L.; Taberlet, P.; Favre, L. and Hausser, J. 1996. Origin and evolution of homologous repeated sequences in the mitochondrial DNA control region of shrews. *Molecular Biology and Evolution* **13**: 31-46.

Wilkinson, G. S. and Chapman, A. M. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics* **128**: 607-617.

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.

Acknowledgements

So many people have contributed their time, expertise and support to help me complete this thesis. Firstly I would like to thank my two fantastic supervisors, Drs Stephen Sarre and Phil Cowan, without whom this work would never have begun, let alone been completed. Steve, thank you for guiding me through the highs and lows of lab work, and for your eternal optimism. Phil, thank you for sharing your wealth of knowledge about possums, and for always making yourself available.

This thesis would not have been possible without the numerous possum tissue samples collected from throughout New Zealand and from Australia. A huge thank you goes to everyone involved in collecting these samples. I would especially like to thank Chris Bee for co-ordinating collection of possums in the South Island buffer zones.

I would like to thank Prof. Dave Lambert for steering me in the direction of Molecular Ecology, and for establishing such an excellent facility with the Molecular Ecology lab. Thanks to everyone in the lab who helped me survive the last two and a half years. In particular I would like to thank Niccy Aitken, firstly for providing me with possum mtDNA sequence data, which meant that my PCR's worked from the very first, and secondly for teaching me how to make a PCR work in the first place! I cannot thank you enough for all your practical assistance in the lab. To Jennie Hay (my "unofficial third supervisor"), thank you so much for giving your time so generously, and for guiding me through the jungle of trees - phylogenetic trees that is. Your insightful comments on previous drafts have made this thesis a much better work. To Olly and Pete, thanks for your help with data analysis, and for stimulating conversations on the nature of evolutionary change. To Hillary, thanks for teaching me the SSCP technique; to Quanah, thanks for helping me chop the ears off possum heads; to Leon, thanks for making me laugh, and for the awesome chicken kebabs; and to Lara, thanks for being my tea-break buddy, and for keeping up such a cracking pace throughout your thesis - if I hadn't been trying to keep up with you I might never have finished (even if you did start one month after me and finish six months before me). Most of all, thanks to everyone in the lab for your friendship and support.

A big thank you is owed to the staff and students of the Ecology department, especially Wendy, Matt, Cindy and Scott (thanks for all the card games while we were supposed to be cramming for exams - I still can't believe we all managed to pass!).

I am very grateful to have received financial support from the Animal Health Board (who also provided financial support for much of this project), the New Zealand Federation of University Woman (Manawatu Branch), Massey University, and the J. P. Skipworth Foundation.

Much love and appreciation goes to Craig Pickering. Your love and support over the last two and a half years has meant the world to me. I know I have been a little difficult to live with at times (just a tiny bit), so thanks for sticking by me, and helping me see this through.

Lastly, I'd like to thank my brother (Stu) and my parents (Denis and Rose). Stu, you helped me develop an indomitable attitude when I was young, which has really helped me throughout this thesis. Dad, you instilled in me a genuine interest in science, and an inquiring mind; Mum, you made me passionate about the environment, and gave me the confidence to believe in myself. I love you both.

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

Table of Contents

CHAPTER ONE - GENERAL INTRODUCTION 1
1.1 OVERVIEW 1
1.2 THE POSSUM PROBLEM 1
1.2.1 History of introduction to New Zealand 1
1.2.2 Problems caused by possums in New Zealand 2
1.2.2.1 Agricultural and environmental 2
1.2.2.2 Diseases carried by possums 3
1.3 POSSUM BIOLOGY AND BEHAVIOUR 4
1.3.1 Reproduction 4
1.3.2 Home range movements 5
1.3.3 Dispersal 6
1.3.3.1 Consequences of dispersal for possum control 6
1.3.3.2 Dispersal is sex-biased 9
1.3.3.3 Dispersal is age-biased 10
1.3.3.4 Methods used to study dispersal 10
1.4 GENETIC ANALYSES OF *TRICHOSURUS VULPECULA* 12
1.4.1 Allozymes 12
1.4.2 Microsatellite DNA 13
1.4.3 Minisatellite DNA 14
1.4.4 Mitochondrial DNA 15
1.4.4.1 Cytochrome b 15
1.4.4.2 Control region - VNTRs 15
1.4.4.3 Control region - non-repetitive regions 15
1.5 MITOCHONDRIAL DNA 16
1.5.1 General characteristics 16
1.5.2 The mitochondrial control region 18
1.5.3 Characteristics of an ideal molecular marker 19
1.6 GENETIC ANALYSES OF POPULATION STRUCTURE USING MTDNA 20
1.6.1 Sex-biased dispersal 20
1.6.2 Effects of geographic distance on population structure 21
1.6.3 Effects of barriers on population structure 21
1.6.4 Utilising mtDNA control region markers to assess possum population genetics 22
1.7 PROJECT DESCRIPTION AND JUSTIFICATION 23

CHAPTER TWO - POPULATION GENETIC SURVEY OF POSSUMS IN NEW ZEALAND 25
2.1 INTRODUCTION 25
2.1.1 Taxonomy of possums in Australia 25
2.1.2 Possums in New Zealand 27
2.2 MATERIALS AND METHODS 31
2.2.1 Study populations and DNA extractions 31
2.2.2 Primer design 35
2.2.3 Amplification of mtDNA control region sequences 37
2.2.4 Purification and quantification of amplified DNA 37
2.2.6 Analysis 38

2.3 RESULTS 39
2.3.1 Sequence variation and diversity of mtDNA control region haplotypes of possums from throughout New Zealand 39
2.3.2 Relationship between New Zealand and Australian possums 49
2.4 DISCUSSION 52
2.4.1 Levels of mtDNA variation in New Zealand 52
2.4.2 Genetic structuring of possums in New Zealand 53
2.4.3 Relationship between New Zealand and Australian possums 55
2.5 CONCLUDING REMARKS 57

CHAPTER THREE - USING mtDNA CONTROL REGION MARKERS TO QUANTIFY POSSUM MOVEMENTS ACROSS BUFFER ZONES 58

3.1 INTRODUCTION 58
3.2 MATERIALS AND METHODS 60
3.2.1 Study populations and DNA extractions 60
3.2.2 Primer design 62
3.2.3 Amplification of mtDNA control region sequences 65
3.2.4 Single-stranded conformation polymorphism (SSCP) analysis 67
3.2.5 Confirmation of haplotypes detected by SSCP 68
3.2.6 Analysis 68
3.3 RESULTS 69
3.3.1 Detection of haplotypes by PCR-SSCP - Pilot Study 69
3.3.2 Detection of Haplotypes by PCR-SSCP - Buffer Zone Study 73
3.4 DISCUSSION 75
3.4.1 Development of SSCP technique 75
3.4.2 Inferring structure and movement patterns from mtDNA variation 76
3.5 CONCLUDING REMARKS 79

CHAPTER FOUR - DISCUSSION AND CONCLUSIONS 80

4.1 SYNTHESIS 80
4.2 INDIRECT METHODS FOR ESTIMATING POSSUM MOVEMENT 81
4.3 SSCP AS A METHOD FOR SURVEYING LARGE NUMBERS OF INDIVIDUALS 83
4.4 FUTURE DIRECTIONS 84

APPENDIX ONE 86

APPENDIX TWO 87

REFERENCES 90

List of Figures

CHAPTER ONE - GENERAL INTRODUCTION

1.1 Tb vector risk areas in New Zealand....8
1.2 Map of mtDNA genome of the American opossum....17

CHAPTER TWO - POPULATION GENETIC SURVEY OF POSSUMS IN NEW ZEALAND

2.1 Distribution of *Trichosurus vulpecula* in Australia....26
2.2 Spread of *Trichosurus vulpecula* in New Zealand (1858-1996)....28
2.3 Possum colour-phase distribution map....29
2.4 Sampling sites for collection of possum tissue in New Zealand....32
2.5 Sampling sites for collection of possum tissue in Australia....33
2.6 Primer map for primers Tv5'F, Tv5'R, Tv3'F and Tv3'R....36
2.7 Haplotype frequencies at all New Zealand sites sampled....41
2.8 Discovery curve of mtDNA control region haplotypes....43
2.9 Neighbour-joining tree of New Zealand possums showing the haplotype and coat colour of each individual surveyed....45
2.10 a: Neighbour-joining tree of New Zealand mtDNA haplotypes; b: Maximum parsimony tree of New Zealand mtDNA haplotypes....47
2.11 Parsimony network of New Zealand mtDNA haplotypes....48
2.12 a: Neighbour-joining tree of New Zealand and Australian mtDNA haplotypes; b: Maximum parsimony tree of New Zealand and Australian mtDNA haplotypes....50
2.13 Parsimony network of New Zealand and Australian haplotypes....51

CHAPTER THREE - USING mtDNA CONTROL REGION MARKERS TO QUANTIFY POSSUM MOVEMENTS ACROSS BUFFER ZONES

3.1 Map of the South Island showing the location of the two buffer zones....61
3.2 Position of variable nucleotide sites in the mtDNA control region of brushtail possums in New Zealand....63
3.3 Primer map of for primers HPTYP1, HPTYP2, SSCP1 and SSCP3....66
3.4 Factors that affect mutation detection in SSCP gels....70
3.5 SSCP gel of all seven mtDNA haplotypes found in New Zealand possums....72

List of Tables

CHAPTER ONE - GENERAL INTRODUCTION

1.1 Published dispersal estimates for possums in New Zealand....7

CHAPTER TWO - POPULATION GENETIC SURVEY OF POSSUMS IN NEW ZEALAND

2.1 Location of possum tissue sampling sites in New Zealand and Australia, and number of individuals collected at each site....34
2.2 Polymorphic sites in the mtDNA control region of possums in New Zealand....40
2.3 Haplotype frequencies of the New Zealand possum populations surveyed....42
2.4 Pairwise distance comparisons between all haplotypes sampled....46

CHAPTER THREE - USING mtDNA CONTROL REGION MARKERS TO QUANTIFY POSSUM MOVEMENTS ACROSS BUFFER ZONES

3.1 Polymorphic sites in Hypervariable region A of possums in New Zealand....64
3.2 Haplotypes detected, and time needed to separate bands, in SSCP gels with various mtDNA fragments....72
3.3 Number of individuals surveyed, and the percentage of black individuals, at each of the eight buffer zone locations....74
3.4 Pairwise ϕ_{ST} comparisons of possums at each of the eight buffer zone locations....75