

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

---

# NUCLEAR AND MITOCHONDRIAL DNA EVOLUTION IN ADÉLIE PENGUINS: STUDIES OF MODERN AND ANCIENT POPULATIONS

---

A thesis presented in partial fulfillment of the requirements for the degree of **Doctor of  
Philosophy (PhD) in Genetics**

Allan Wilson Centre for Molecular Ecology and Evolution  
Institute for Natural Sciences  
Massey University  
Auckland, New Zealand

**GABRIELLE ANGELA BEANS PICÓN**

**2012**



Teatro mágico - sólo para locos

La entrada cuesta la razón

- Hermann Hesse





# ABSTRACT

---

The Adélie penguin of Antarctica (*Pygoscelis adeliae*) breeds on the Antarctic continent and on offshore islands. Its evolutionary history has been, and its current biology remains, dependent on a range of climate variables. Over geological time, glacial warming and cooling periods have resulted in Adélie penguin populations decreasing and expanding. Therefore, understanding Adélie penguin population dynamics at a genetic level can provide insights into how the species responds to changing climates, one reason why Adélie penguins are an important natural model species. In addition, sub-fossil bone deposits of this species below modern and abandoned colonies provide an excellent source of ancient DNA that can bring a temporal dimension to population studies of the species. In combination, these attributes enable us to address some fundamental questions regarding evolutionary change.

Making use of known mitochondrial DNA mutation rates and current population sizes, a positive and significant correlation between population size and modern mitochondrial control region diversity was detected. This finding supports the use of mitochondrial DNA for population inferences. Effective population sizes of breeding colonies are shown to have increased since the late Pleistocene. To extend current tools available for understanding Adélie penguins, six nuclear intron loci were recovered from a wide range of introns that can be applied to population genetics and phylogenetic studies of penguins. Five introns were used to investigate the persistence of the mitochondrial Antarctic (A) and Ross Sea (RS) lineages. No evidence for the existence of these lineages was found in the nuclear loci sequenced. A signature of historical population expansion, preceding the mitochondrial one, was detected. The utility of four introns in resolving penguin phylogenetic signals was also determined. Non-coding nuclear sequence of one intron were obtained from ancient sub-fossil remains of Adélie penguins using multiplex PCR enrichment, followed by second-generation sequencing of a barcoded library. A shift in haplotype frequencies was detected between ancient and modern intron sequences in Adélie penguins, despite a small sample size. In the future, advancing the current methodologies and extending sampling to additional introns as well as older samples, is likely to provide a new level of understanding of this remarkable species.



# ACKNOWLEDGEMENTS

---

It's hard to believe so much time has gone by since I first landed in New Zealand, eager to begin an adventure on different levels. Certainly, I have experienced more than I would have anticipated on that sunny day, May 31<sup>st</sup>, 2006, when I thought the cab driver was messing with me by taking such a bizarre suburban route from the airport to the lab at Massey in Albany. It's the end of one road now, finally, and my PhD adventure is winding down as I write these words. There are, of course, an innumerable number of people I would like to thank in great detail for their support, both academically and on a personal level. I suppose everyone feels this way!

First, and foremost, I owe great thanks to my supervisor, David Lambert, for a great number of things; for offering me the chance to come to New Zealand, for letting me explore my own ideas for different projects, for encouraging me to support the All Blacks, for helping me find positive approaches to my writing when I was deep in thesis-end negativity. Thank you, as well, and Sherene and Christine, for welcoming me into your home in Brisbane and giving my thesis a jump-start when it most needed it.

Thanks to my co-supervisor Austen Ganley, for useful discussions and moral support when I needed it, among other things. Special thanks to Leon Huynen and Sankar Subramanian, who gave me invaluable help, particularly in my last panicked moments when I unreasonably needed things done quickly. You really came through for me, and I greatly appreciate it. Thanks also to my examiners for helpful comments and for giving me the PhD in the end!

Thanks to everyone who contributed to different aspects of this research, either directly or through previous work on Adélie penguins. Thanks to everyone who





collected samples before my arrival (though I do wish I could have gone and collected some myself, it's incredibly helpful to have freezers and fridges full of bones and



blood when you start off), and everyone who helped produce the mitochondrial DNA sequences I analyzed for part of this thesis. Thanks also to everyone involved in radio-carbondating Adélie subfossil bones. Thanks to Phil Lyver for collaborating with us and providing demographic Adélie data, as well as for being great to chat to.

Thanks to Michael Knapp for helping me get to grips with tagging a load of ancient multiplexed introns for my FLX run and for being a great host at Otago University, and of course thanks to Lisa Matisoo-Smith for welcoming me to her group for those few weeks I was visiting. Thanks to Allan Baker and Oliver Ryder for “other” penguin samples and DNA. Thanks especially to Oliver for extracting DNA for me twice due to NZ customs mysteriously keeping one DNA shipment! Thanks to Tim for showing me round the Australian bush and diving.

Thanks to the Allan Wilson Centre, Massey University, the New Zealand Postgraduate Study Abroad Award, Massey University Institute of Molecular Biosciences and Massey University Institute of Natural Sciences for funding my PhD scholarship and conference travel (Wellington, Hawaii, Christchurch, Kaikoura, Barcelona oh my!).

I can't possibly fit in here all the friends from afar that have been there for me, and the countless friends I have made since moving to New Zealand. Every one of them has played a part in making my life here memorable in every way imaginable. From hot tub parties to laid back barbecues, theme parties to Pohutukawa-filled wild Christmases, from Tongan beach escapes to South Island winter and summer adventures, from mountain biking to diving to hiking to just simply walking around the East Coast Bays, from east coast sunrises to epic west coast sunsets... I have loved making New Zealand my home and you are all the main reason it feels that way. Thank you! Special thanks to Katie, Jyothsna, Martina, Hayley, Eli, Jarod, Phil,

all flatmates past and present, AUUC, Jarod, Arapeta & Ra (for giving me my own taonga) and everyone who spoke to me in Building 11 since the beginning. Thanks to anyone who came to visit while I was here.

Yes, of course, thanks to my family, who from the beginning were nothing but supportive (though I do recall my father trying to get me a PhD position in Austria so I wouldn't leave Europe). Thanks for not minding too much that I was literally on the other side of the world! Thanks for supporting me in difficult times (there have been a few, I can't lie), and for coming all the way out here to try and understand why I like it so much (not to mention sustaining the NZ economy by purchasing every possible kind of souvenir). I am incredibly lucky to have so much love in my life, and though I may be a bit difficult sometimes, I never take it for granted. Everything I am is thanks to you.

Eric, you came into this story near the end, during the most difficult and also the most joyous moments. Thank you for putting up with me during my thesis meltdowns, thank you for keeping me going, encouraging me, and always bringing hope and happiness with you.



Finally, thanks, beautiful, green, ocean-fringed Aotearoa. No matter where I end up, I will love this quirky little country forever. Verde que te quiero verde!



## **Table of Contents**

<b>ABSTRACT</b>	<b>I</b>
<b>ACKNOWLEDGEMENTS</b>	<b>II</b>
<b>TABLE OF CONTENTS</b>	<b>V</b>
<b>LIST OF FIGURES</b>	<b>X</b>
<b>LIST OF TABLES</b>	<b>XIII</b>
<b>CHAPTER ONE</b>	<b>1</b>
<b>INTRODUCTION</b>	
<b>1.1 THESIS STRUCTURE</b>	<b>1</b>
<b>1.2 ADÉLIE PENGUINS</b>	<b>2</b>
1.2.1 MOLECULAR ECOLOGY AND EVOLUTION IN ADÉLIE PENGUINS	4
<b>1.3 MOLECULAR MARKERS</b>	<b>6</b>
<b>1.4 MITOCHONDRIAL DNA</b>	<b>8</b>
1.4.1 THE RELATIONSHIP BETWEEN MITOCHONDRIAL DNA DIVERSITY AND POPULATION SIZE	10
1.4.2 CRITICISM OF MTDNA IN POPULATION GENETICS AND PHYLOGENETICS	13
<b>1.5 NUCLEAR INTRONS</b>	<b>14</b>
1.5.1 FOUR GROUPS OF INTRONS	15
1.5.2 SPLICEOSOMAL INTRON EVOLUTION	16
1.5.3 USING INTRONS IN PHYLOGENETICS AND POPULATION GENETICS	20
1.5.4 NUCLEAR INTRONS FOR PENGUINS	23
<b>1.6 EVOLUTIONARY RATES</b>	<b>23</b>
1.6.1 METHODS OF CALCULATING EVOLUTIONARY RATES	24
1.6.2 APPARENT TIME-DEPENDENCY OF RATES	25
<b>1.7 INTRON EVOLUTIONARY RATES AND THE POTENTIAL OF ANCIENT DNA TECHNIQUES</b>	<b>26</b>

<b>1.8</b>	<b>AIMS OF THIS PhD</b>	<b>29</b>
<b>1.9</b>	<b>REFERENCES</b>	<b>30</b>

---

## **CHAPTER TWO** **41**

---

### GENETIC DIVERSITY AND EFFECTIVE POPULATION SIZE OF ADÉLIE PENGUINS

---

<b>2.1</b>	<b>ABSTRACT</b>	<b>41</b>
<b>2.2</b>	<b>INTRODUCTION</b>	<b>42</b>
<b>2.3</b>	<b>MATERIAL AND METHODS</b>	<b>44</b>
2.3.1	ANNUAL COUNT OF ADÉLIE PENGUIN BREEDING PAIRS	44
2.3.2	SAMPLES, DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING	45
2.3.3	SUMMARY STATISTICS AND POPULATION STRUCTURE (AMOVA)	47
2.3.4	GENETIC ESTIMATES OF LONG-TERM EFFECTIVE POPULATION SIZES OF COLONIES OF ADÉLIE PENGUINS IN THE ROSS SEA	48
<b>2.4</b>	<b>RESULTS</b>	<b>49</b>
2.4.1	SUMMARY STATISTICS AND POPULATION STRUCTURE	49
2.4.2	ESTIMATES OF GENETIC DIVERSITY ( $\theta$ ) AND FEMALE EFFECTIVE POPULATION SIZE ( $N_{EF}$ )	51
<b>2.5</b>	<b>DISCUSSION</b>	<b>55</b>
<b>2.6</b>	<b>REFERENCES</b>	<b>61</b>

---

## **CHAPTER THREE** **65**

---

### EFFECTIVE POPULATION SIZE OF THE EXTINCT HUIA

---

<b>3.1</b>	<b>ABSTRACT</b>	<b>65</b>
<b>3.2</b>	<b>INTRODUCTION</b>	<b>66</b>
<b>3.3</b>	<b>MATERIAL AND METHODS</b>	<b>69</b>
3.3.1	SAMPLES	69
3.3.2	ANCIENT DNA METHODS	71
3.3.3	HUIA MITOCHONDRIAL HYPERVARIABLE REGION SEQUENCES	72
3.3.4	ESTIMATING GENETIC DIVERSITY AND EFFECTIVE POPULATION SIZE IN HUIA	72
<b>3.4</b>	<b>RESULTS</b>	<b>73</b>
3.4.1	ESTIMATING GENETIC DIVERSITY AND POPULATION SIZE OF HUIA	73
<b>3.5</b>	<b>DISCUSSION</b>	<b>75</b>
<b>3.6</b>	<b>REFERENCES</b>	<b>78</b>

---

**CHAPTER FOUR** **81**

---

**INTRON RECOVERY IN ADÉLIE PENGUINS**

---

<b>4.1</b>	<b>ABSTRACT</b>	<b>81</b>
<b>4.2</b>	<b>INTRODUCTION</b>	<b>82</b>
<b>4.3</b>	<b>METHODS</b>	<b>85</b>
4.3.1	CHOOSING INTRON MARKERS FOR ADÉLIE PENGUINS	85
4.3.2	DNA EXTRACTIONS	86
4.3.3	SCREENING INTRON MARKERS IN MODERN ADÉLIE PENGUINS	86
<b>4.4</b>	<b>RESULTS</b>	<b>92</b>
4.4.1	LITERATURE AND NCBI SEARCH	92
4.4.2	PRIMER SCREEN AND INITIAL INTRON SEQUENCING RESULTS	93
<b>4.5</b>	<b>DISCUSSION</b>	<b>97</b>
<b>4.6</b>	<b>REFERENCES</b>	<b>101</b>

**CHAPTER FIVE** **107**

---

**USING INTRONS TO ELUCIDATE ADÉLIE PENGUIN POPULATION  
GENETICS AND PENGUIN PHYLOGENY**

---

<b>5.1</b>	<b>ABSTRACT</b>	<b>107</b>
<b>5.2</b>	<b>INTRODUCTION</b>	<b>108</b>
<b>5.3</b>	<b>METHODS</b>	<b>110</b>
5.3.1	DNA EXTRACTIONS	110
5.3.2	PCR AND DIRECT SEQUENCING	112
5.3.3	SEQUENCE ANALYSIS AND PHASING OF INTRONS	113
5.3.4	ADÉLIE POPULATION GENETIC ANALYSIS	114
5.3.5	PENGUIN INTRON PHYLOGENETICS	117
<b>5.4</b>	<b>RESULTS</b>	<b>119</b>
5.4.1	ADÉLIE PENGUIN INTRON ANALYSES	119
5.4.2	PENGUIN PHYLOGENETIC ANALYSES	127
<b>5.5</b>	<b>DISCUSSION</b>	<b>131</b>
5.5.1	ADÉLIE PENGUIN INTRON POPULATION GENETICS	132
5.5.2	PENGUIN PHYLOGENY	135
5.5.3	CONCLUSIONS	137
<b>5.6</b>	<b>REFERENCES</b>	<b>138</b>

---

**CHAPTER SIX** **143**

---

**RECOVERING ANCIENT NUCLEAR INTRONS OF ADÉLIE PENGUINS USING  
SECOND-GENERATION SEQUENCING**

---

<b>6.1</b>	<b>ABSTRACT</b>	<b>143</b>
<b>6.2</b>	<b>INTRODUCTION</b>	<b>144</b>
<b>6.3</b>	<b>METHODS</b>	<b>147</b>
6.3.1	DNA EXTRACTIONS FROM BONE	147
6.3.2	PCR VERIFICATION OF DNA EXTRACTIONS	148
6.3.3	DESIGNING INTERNAL PRIMERS FOR ANCIENT DNA WORK	150
6.3.4	DIRECT MULTIPLEX PCR FLX SEQUENCING METHODOLOGY	151
6.3.5	ANALYTICAL METHODS	155
<b>6.4</b>	<b>RESULTS</b>	<b>158</b>
6.4.1	FLX OUTPUT, ASSEMBLY AND COVERAGE	158
6.4.2	ANALYSIS OF MODERN AND ANCIENT AK115 SEQUENCES	161
6.4.3	BLAST RESULTS OF CONTAMINANT READS	166
<b>6.5</b>	<b>DISCUSSION</b>	<b>171</b>
6.5.1	DIRECT MULTIPLEX FLX SEQUENCING	171
6.5.2	ANCIENT ADÉLIE ADENYLATE KINASE INTRON 5 SEQUENCES	173
6.5.3	CONTAMINANT SEQUENCES	174
6.5.4	CONCLUSIONS	176
<b>6.6</b>	<b>REFERENCES</b>	<b>178</b>

---

**CHAPTER SEVEN** **183**

---

**CONCLUSIONS AND PERSPECTIVES**

---

<b>7.1</b>	<b>INTRODUCTION</b>	<b>183</b>
<b>7.2</b>	<b>THESIS SUMMATION</b>	<b>184</b>
<b>7.3</b>	<b>FUTURE PERSPECTIVES</b>	<b>187</b>
<b>7.4</b>	<b>REFERENCES</b>	<b>189</b>

---

**I APPENDIX ONE** **191**

---

**THE MOLECULAR ECOLOGY OF THE EXTINCT NEW ZEALAND HUIA**

---

---

**II APPENDIX TWO** **203**

SUPPLEMENTARY MATERIAL CHAPTER FIVE: INDIVIDUAL  
PHYLOGENETIC TREES

---

**III APPENDIX THREE** **209**

---

SUPPLEMENTARY MATERIAL FOR CHAPTER SIX: EXTENDED METHODS

---

**III.1 DIRECT SEQUENCING OF ANCIENT MYELIN PROTEOLIPID PROTEIN INTRON  
FOUR 210**

**III.2 FLX TAGGING PROTOCOL AND LIBRARY QUANTIFICATION 212**

**IV APPENDIX FOUR 221**

---

DRC AUTHOR CONTRIBUTION FORMS

---





## List of Figures

<b>Figure 1.1: Phylogenetic representation of modern penguins, taken from (BAKER <i>et al.</i> 2006).</b>	<b>3</b>
<b>Figure 1.2: Schematic drawing of different marker classes, their relative variability and adequacy for different research questions.</b>	<b>7</b>
<b>Figure 1.3: The Adélie penguin mitochondrial genome.</b>	<b>11</b>
<b>Figure 1.4: U2-type spliceosomal intron splicing mechanism.</b>	<b>15</b>
<b>Figure 1.5: Different theories for the evolution of introns, a) introns late theory (L) and b) introns early (E) and first (F) theories.</b>	<b>17</b>
<b>Figure 1.6: Structure of the human beta-fibrinogen gene, including both coding (exon) and noncoding (intron) regions.</b>	<b>21</b>
<b>Figure 2.1: The distribution of Ross Sea Adélie penguin colonies (1 – 15) from which samples were used in this study, along with a photograph (courtesy of K. Barton) used for counting number of breeding pairs from the colony at Cape Bird.</b>	<b>46</b>
<b>Figure 2.2: Schematic representation of the late Pleistocene migration of Adélie penguins into the Ross Sea of Antarctica, together with estimation times for the common ancestor of the current populations.</b>	<b>58</b>
<b>Figure 3.1: Extreme reverse sexual bill dimorphism in Huia.</b>	<b>67</b>
<b>Figure 3.2: Provenance of Huia samples used in this study.</b>	<b>69</b>
<b>Figure 3.3: DNA nucleotide variation in 199bp of the mitochondrial hypervariable region (HVRI) among 21 Huia.</b>	<b>73</b>
<b>Figure 3.4: Most probable mitochondrial diversity (<math>\theta</math>) estimate from LAMARC.</b>	<b>74</b>
<b>Figure 3.5: Effect of generation time and mutation rate on the population size estimate</b>	<b>77</b>
<b>Figure 4.1: Distribution of Adélie penguin samples used in this study</b>	<b>87</b>
<b>Figure 4.2 Hypothetical length variant heterozygote electropherogram read showing mixed peaks as a result of a 1bp indel (marked in red).</b>	<b>92</b>
<b>Figure 4.3 2% agarose gel showing the six intron markers that produced high quality sequence well in Adélie penguins.</b>	<b>94</b>
<b>Figure 4.4 Adenylate kinase intron 5 length variant heterozygote.</b>	<b>95</b>
<b>Figure 4.5 Primer positions and amplicon sizes for Adenylate kinase intron 5 external and internal primers (not to scale).</b>	<b>96</b>
<b>Figure 4.6 Variable sites of introns sequenced in the Adélie penguin as compared to available sequences from other penguin species.</b>	<b>96</b>

Figure 5.1 Phylogenetic representation of extant penguin genera and divergence times.	109
Figure 5.2 Haplotypes found for four introns in Adélie penguins.	120
Figure 5.3 Haplotypes found for <i>Ak1i5</i> in Adélie penguins.	121
Figure 5.4 Mismatch distributions for five intron loci in Adélie penguins.	123
Figure 5.5 Haplotype networks constructed using sequence data from four introns of Adélie penguins.	126
Figure 5.6 Haplotype network constructed using sequence data from <i>AK1i5</i> of Adélie penguins.	127
Figure 5.7 Unrooted Bayesian modern penguin phylogenetic consensus tree for the concatenated four intron dataset (1926 bp incl. gaps).	129
Figure 5.8 Rooted Bayesian modern penguin phylogenetic consensus tree for the concatenated <i>AK1i5/ODC6</i> dataset (1216 bp incl. gaps).	130
Figure 6.1: Overview of direct multiplex sequencing of ancient Adélie penguin nuclear intron products.	146
Figure 6.2: The age and geographical distribution of subfossil Adélie penguin bones used in this study.	149
Figure 6.3: Multiplex primer positions and groupings.	153
Figure 6.4: FLX read distribution across tags and replicates.	159
Figure 6.5: Defining nucleotide changes for haplotypes of modern and ancient <i>AK1i5</i> sequences.	164
Figure 6.6: Temporal haplotype network for modern and ancient Adélie <i>AK1i5</i> sequences.	165
Figure 6.7: Distribution of BLAST hit results for FLX sequencing reads.	168
Figure 6.8: Distribution of significant BLAST hits for FLX sequencing reads.	169
Figure II.1 Unrooted Bayesian modern penguin phylogenetic consensus tree for locus <i>UCHL3</i> .	204
Figure II.2: Rooted Bayesian modern penguin phylogenetic consensus tree for locus <i>AK1i5</i> .	205
Figure II.3: Rooted Bayesian modern penguin phylogenetic consensus tree for locus <i>MPP4</i> .	206
Figure II.4: Rooted Bayesian modern penguin phylogenetic consensus tree for locus <i>MPP4</i> ..	207
Figure II.5: UPGMA phylogenetic tree for the four concatenated intron-only dataset.	208
Figure III.1: Schematic representation of positions of <i>MPP4</i> primers.	211

<b>Figure III.2: <i>MPP4</i> direct sequencing results in four fragments from ancient Adélie subfossil bone samples.</b>	<b>212</b>
<b>Figure III.3: Example of a tagged target sequence prior to the adapter fill-in step.</b>	<b>213</b>
<b>Figure III.4: 3% agarose gel showing the result of a ligation test of one adapter.</b>	<b>215</b>
<b>Figure III.5: Consensus ancient Adélie <i>AKI5</i> sequences obtained from FLX sequencing.</b>	<b>220</b>



## List of Tables

Table 2.1 Summary statistics for <i>HVRI</i> in fifteen Adélie penguin colonies	50
Table 2.2: AMOVA results.	51
Table 2.3: Pairwise $\phi_{st}$ results.	52
Table 2.4: Estimates of population sizes from population counts together with genetic diversity in colonies of Adélie penguins.	53
Table 3.1: Huia samples used in this study.	70
Table 3.2: Long-term population size estimates of Huia based on mitochondrial hypervariable region diversity. Reproduced with permission from Lambert <i>et al</i> (2009).	75
Table 4.1 Details of primer pairs tested in Adélie penguin samples from the Ross Sea, Antarctica.	84
Table 4.2 PCR conditions and annealing temperature ranges tested during optimization runs for 26 primer pairs in modern samples.	88
Table 4.3 PCR program details for modern samples.	88
Table 4.4 Results of PCR condition testing intron screen in modern Adélie penguins.	90
Table 5.1 Adélie penguin sample provenance, together with mtDNA lineage and intron haplotypes	111
Table 5.2 Penguin species sequenced for four introns	112
Table 5.3 Summary statistics for five intron loci in Adélie penguins.	122
Table 5.4 Results of the mismatch analysis for five intron loci in Adélie penguins and $\theta$ estimates.	125
Table 5.5 Effective population size estimates for five intron loci in Adélie penguins	125
Table 6.1: Multiplex primer groupings and information for the three different introns that were the subject of this study.	152
Table 6.2 Coverage and distribution of FLX reads for adenylate kinase intron 5	160
Table 6.3: Modeltest results for modern and ancient AK1i5 Adélie datasets	163
Table 6.4: Summary statistics and neutrality tests for modern and ancient AK1i5 Adélie datasets	163
Table 6.5: Ancient and Modern Samples included in Analyses	164
Table III.1 Internal primers for <i>MPP4</i>	210
Table III.2: Barcoded Adapters used for DMPS FLX Titanium Sequencing	216