Biomedical Sciences

An assessment of genetic diversity and inbreeding in the Noisy Scrub-bird (*Atrichornis clamosus*) using microsatellite and Major Histocompatibility Complex loci

Saul Jesse Cowen

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

November 2014

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date: 23/11/2014

Table of contents

Acknowledgements 1
Abstract 5
List of abbreviations 9
Chapter 1 – Background 11
1.1 Threats to global biodiversity 11
1.2 Biodiversity conservation and genetics 13
1.2.1 Genetic diversity as biodiversity 13
1.2.2 Conserving genetic diversity in island bird species 21
1.3 Translocations and biodiversity conservation 25
1.3.1 Translocations as conservation management strategy 25
1.3.2 Translocations and genetic diversity 27
1.4 Study species 31
1.4.1 Noisy scrub-bird 31
1.4.2 Barrow Island white-winged fairy-wren 42
1.4.3 Spinifexbird 46
Chapter 2 – Materials and methods 49
2.1 Population census 49
2.1.1 Aims of surveys for census 49
2.1.2 Survey locations 50
2.2 Capture and tissue sampling 51
2.2.1 Aims of tissue sampling 51
2.2.2 Capture methodology 52
2.2.3 Tissue sampling methodology 54
2.2.4 Summary of capture work 55
2.3 DNA extraction 55
2.3.1 DNA extraction methodology (blood) 55
2.3.2 DNA extraction methodology (feathers) 56
2.3.3 Gel extraction methodology 57
2.4 Polymerase chain reaction 57
2.4.1 Polymerase chain reaction methodology 57
2.5 Primer design 58

2.5.1 Microsatellite primer design methodology 58
2.6 Capillary electrophoresis 59
2.6.1 Capillary electrophoresis methodology 59
2.7 Transformation of competent cells 60
2.7.1 Ligation of PCR product into plasmid vector 60
2.7.2 Transformation of competent cells 60
2.7.3 Clone selection and plasmid extraction for sequencing 61
Chapter 3 – Microsatellite discovery and characterisation 63
3.1 Introduction 63
3.2 Methodology 65
3.2.1 DNA extraction and preparation for 454 65
3.2.2 Construction of gDNA library using 454 shotgun pyrosequencing 65
3.2.3 Screening and interrogation of 454 data for microsatellite loci 66
3.2.4 Primer design and detection of polymorphic microsatellite loci 67
3.2.5 Fragment analysis of polymorphic microsatellites 67
3.3 Results 67
3.3.1 DNA extraction and second-generation sequencing 67
3.3.2 Comparison of microsatellite motif abundances in different studies ... 68
3.3.3 Characterisation of tetra-nucleotide microsatellite loci 74
3.4 Discussion 75
Chapter 4 – Microsatellite diversity in three genetic populations of noisy scrub-bird (*Atrichornis clamosus)* 80
4.1 Introduction 80
4.2 Methodology 86
4.2.1 Analysis of microsatellites by capillary electrophoresis 86
4.2.2 Statistical analysis of microsatellite data 86
4.3 Results 89
4.4 Discussion 100
4.4.1 Intra- and inter-population genetic diversity in MG, MP and BA 100
4.4.2 Implications for conservation and management 106
Chapter 5 – Diversity of the Major Histocompatibility Complex Class II B gene in the noisy scrub-bird 109
5.1 Introduction 109
5.2 Methodology 113

5.2.1 Amplification and sequencing of MHC Class II B loci 113
5.2.2 Analysis of MHC sequence data 116
5.3 Results 119
5.4 Discussion 137
5.4.1 Diversity in the MHC Class II B 137
5.4.2 Implications for conservation.. 140
Chapter 6 – Diversity in Major Histocompatibility Complex Class II B gene and pathogen prevalence in two island bird species 145
6.1 Introduction 145
6.2 Methodology 148
6.3 Results 149
6.4 Discussion 153
Chapter 7 – Discussion 157
7.1 Microsatellite diversity and inbreeding in the noisy scrub-bird 157
7.2 MHC Class II B diversity in the noisy scrub-bird.................................... 159
7.3 MHC Class II B diversity in Barrow Island birds 161
7.4 Implications for conservation management 163
7.5 Future directions... 166
Appendices 169
Appendix i - List of noisy scrub-bird samples 169
Appendix ii - List of black-and-white fairy-wren & spinifexbird samples.......... 172
Appendix iv - Qiagen DNeasy Blood and Tissue Kit Protocol: Purification of Total DNA from Animal Blood (Spin-Column Protocol) (adapted for nucleated erythrocytes) (Qiagen, 2006) 177
Appendix v - QIAquick Gel Extraction Kit Protocol (using a microcentrifuge) (Qiagen, 2008)... 180
Appendix vi - Polymerase Chain Reaction parameters for amplifying short-tandem repeats and Major Histocompatibility Complex Class II B exon 2 - intron 2 - exon 3.. 183
Appendix vii - Protocol for Ligations Using pGEM-T Easy Vector and the 2X Rapid Ligation Buffer (Promega, 2010) 185
Appendix viii - Bioline ElectroSHOX Competent Cells - Suggested Transformation Procedure for Optimal Results - (Bioline, 2013) 187

Appendix ix - Favorgen FavorPrepPlasmid DNA Extraction Mini Kit (Favorgen, 2011)..188
References 191

Acknowledgements

The author would like to acknowledge the Menang people, the traditional owners of the land where the noisy scrub-bird (Tjimiluk) now occurs, including Toolberup (Two Peoples Bay) and Yoolberup (Mt Manypeaks).

This thesis is dedicated to the memory of Lyndsay Eileen Cowen. Without inheriting her tenets of comittment, hard-work, self-belief and a dedication to one's passion, my path in life would never have led me to this place. Emotionally, spiritually and financially this work is as much hers as it is mine.

The author would like to thank the Western Australian Department of Parks and Wildlife (previously Department of Environment and Conservation) for its support during this study. In particular, the major contribution of the South Coast Threatened Birds Recovery Team has been instrumental in the conception and execution of this project. Allan Burbidge, Sarah Comer and Alan Danks must be singled out for their major contributions to this work, which have been vital in shaping and focussing its direction. On a personal level, without Sarah's remarkable enthusiasm, irrational optimism and a belief that anything is possible, this thesis may never have come to fruition.

In addition, I am indebted to my good friends and work colleagues on the Integrated Fauna Recovery Program (Louisa Bell, Abby Berryman, Lucy Clausen, Jeff Pinder Jon Pridham and Cameron Tiller) for their friendship, support and difficult questions during the beginning, middle and end of this thesis. The final year of my doctorate tested me with the biggest challenges of my life so far and for the kindness and support that my DPaW comrades have shown me I will always be grateful. In addition, thank you to Dave Algar for continuing to wonder why I haven't finished my PhD yet!

At the Curtin University School of Biomedical Sciences, I would like to thank my supervisors David Groth and John Wetherall first of all, but also Ricardo Mancera, Cyril Mamotte and Elizabeth Watkin for their support. Like Sarah, David's belief that anything is possible was instrumental in the completion of this project and I would like to thank David (and John) for their patience with a zoologist turned

geneticist. Thanks must also be extended to Eleanor (Nell) Morgan who greatly assisted with experimental design and execution of the use of phylogenetic trees.
There is a plethora of members of Lab 140 that I would like to thank but the first must be Danielle Giustiniano, Ryan Harris and Sharon Siva with whom I have shared the successes, disappointments and stupid questions this study has brought. I would also like to thank Mark Castalanelli and Jason Ledger for their expertise and advice at the start, as well as Charlene Babra Waryah, Chris Bryan, Rhys Cransberg, Jaden Elphick, Laurton McGurk and Rob Steuart for their advice, assistance and good banter during my time in 140. I also cannot fail to mention Kieran Mulroney who provided invaluable assistance in the epic task of nearly 1000 plasmid extractions!
The contribution of Richard Allcock and Nina Kresoje at the LotteryWest State Biomedical Facility-Genomics was invaluable in providing 454 sequencing for the NSB microsatellite work. Without their expertise and advice, the microsatellite portion of this study may not have been nearly as smooth.
Thanks also to Dr. David Edmonds (WA) and Dr. John Chitty (UK) for their valuable veterinary insights into pathogens and the MHC in birds.
Obtaining significant funding for conservation genetics research can be tricky but the generous support of Birdlife Australia through the Stuart Leslie Bird Research Award and Prof. Allen Keast Research Award has allowed this project to reach its full potential, whilst validating the conservation value of the work itself for the preservation of this special bird for generations to come. In particular I would like to thank James O'Connor and Margaret Quixley for their fantastic support and encouragement throughout. In addition, the Guild of Mercers' Scholars Charitable Fund has been very generous to various projects over the years and made a significant contribution to the purchase of essential field equipment for work on Bald Island.
In no particular order I'd like to thank Ross Smith, Ciska Du Plessis, Casey Ridder and everyone else at Macpac Perth; Peter Collins, Darren 'Stretch' Little, Tony Friend, Tim Button, Ben Atkinson, Cassidy Newland, Toby Deadman, Don Crecy, Troy Makan, Bridget Makan (née Wrenn), Tim Doherty, Alex Saka, Mark Dixon, Brent Nottage, Brian Goodwin and Kimiora McCarthy for their help with missions to Bald Island as well as all the other staff and volunteers who have assisted with NSB work on the mainland as well; coffee buddy, fellow 'bird-nerd' and Curtin PhD 'inmate' Tegan Douglas; Paul 'Scratch' Jansen, Brent Barrett, Richard Maloney

(DoC, New Zealand) and David and Karen Waters (Great Bustard Group, UK) who have helped shape the path that I've travelled in the last 12 years; Dr. Yvonne Collingham and Dr. Sean Twiss at Durham University for helping to get my research career off and running.

Finally, there aren't sufficient words to describe the myriad contributions of my parents, Lyndsay and Lewis Cowen, my wonderful wife Dearne Cowen and my daughter Rachael. Their love, support and encouragement has been the edge I've needed throughout this chapter of my life, and I'm looking forward to beginning the next one.

Abstract

An assessment of genetic diversity and inbreeding in the Noisy Scrub-bird (Atrichornis clamosus) using microsatellite and Major Histocompatibility Complex loci

The main aim of this project has been to use molecular markers to investigate the degree of genetic diversity in the unique remnant population of the noisy scrub-bird (NSB) in Western Australia for a conservation management program for this threatened species. Birds from an original founding population and from two other populations derived from the original population by translocations, approximately 30 and 20 years previously, were assessed. The management program for the NSB was initiated due to the small size of the original population and its innate vulnerability to stochastic events such as fire, non-native predators or disease.

Blood and feather samples were collected from individuals from each of the three populations. DNA was extracted from these samples for subsequent discovery of genetic markers using molecular techniques. Short tandem repeat loci (referred to as microsatellites) have been widely used in genetic diversity studies and were therefore selected for this study. The advent of new generation DNA sequencing technology provides an efficient and cost-effective method for the discovery of these markers, especially in species for which no previous information is available. Consequently second-generation sequencing technology, as implemented in the 454 Life Sciences (Roche) sequencing technology, was used in this study on Noisy Scrub-bird genetic diversity. Approximately 1.1 million genomic sequence reads (averaging 278 base pairs long) were obtained.

Sequence analysis of these sequences identified 2,625 di-nucleotide loci, 989 tri-nucleotides and 1,450 tetra-nucleotide loci. Of these only the tetra-nucleotide loci were further characterised for genetic diversity studies because they are less prone to amplification error and it is easier to discriminate between allelic variants at these loci relative to di-nucleotide loci. A panel of 10 polymorphic tetra-nucleotide loci was

selected for genotyping of DNA samples from individual NSBs from each of the three populations in order to generate allele frequency data for each population.
Results of population genetic analysis using these microsatellite loci highlighted the low genetic diversity across all populations, with a marginally higher allelic diversity in the original remnant population than in the two derived populations. Allelic diversity in general was comparable with a range of avian species that have experienced critical population bottlenecks. Deviation from Hardy-Weinberg Equilibrium was observed for all three populations. However, only the original population and one of the translocated populations produced statistically significant inbreeding values (F_{IS}). Statistically significant differentiation was observed between the two derived populations but not between either of these populations and the original. Evidence for putative null alleles was obtained for two loci. However, confirmation by breeding experiments was not possible and homozygote excess observed in two of the three populations could account for this observation. Allelic frequency data was used to model and estimate past bottleneck sizes, intra-population genetic structure and effective population size. Modelling the size and occurrence bottlenecks was probably compromised by overall low allelic diversity and relatively low numbers of loci, with just one bottleneck predicted (Mt Manypeaks) despite our knowledge of bottlenecks in all three populations. Population structure modelling predicted three demographic clusters but these were within populations, rather than between geographic populations, suggesting no inter-population structure. However, there was some evidence of intra-population structure, which may be important for sourcing founders for future translocations. Estimates for effective population sizes were potentially informative and suggested a relatively small reproductive demographic cohort, which might be expected in a presumed polygynous species. However, due to large variation for the 95% confidence interval, these results should be treated with caution. This was also true for estimates for the number of reproductive founders of the Bald Island population.

A second aspect of this study involved identifying and sequencing DNA from the Class II region of the MHC in the NSB. Priming sequences were identified by testing sequences derived from published reports of Class II MHC loci a in variety of avian and reptilian species. NSB Class II sequence fragments were successfully amplified and these were cloned for subsequent sequencing and alignment. A large number of

sequences for exon 2 to exon 3 from NSB Class II loci were obtained. It was clear that multiple Class II loci were identified for each of the three populations studied and that each locus was polymorphic. Translation and subsequent alignment of the Class II peptides from exons 2 and 3 clearly confirmed the presence of several distinct loci characterised by unique amino acid sequence motifs. This result indicates extensive genetic diversity within the Class II region of the NSB MHC and contrasts with the paucity of genetic diversity observed using microsatellite loci. While this result is consistent with the importance of diversity within the MHC for the expression of immunity against a variety of pathogens, very few pathogens have been detected in the NSB. In the absence of a candidate pathogen, the mechanism driving the MHC Class II B evolution is not apparent in the NSB, despite analyses showing that the observed diversity has resulted from a combination of gene conversion and balancing selection.

Finally, to elucidate the role of pathogen exposure in driving MHC Class II B evolution a search for allelic associations with specific pathogens was undertaken. Like the NSB, the black-and-white fairy-wren (*Malurus leucopterus edouardi*) and the spinifexbird (*Eremiornis carteri*) are Passerine bird species and the populations of these species on Barrow Island in north-west Western Australia have been found to have a high prevalence of pathogens, particularly *Chlamydophila psittaci*. Exon 2 to Exon 3 of the MHC Class II B was successfully amplified and sequenced for a cohort of the population of each species. Sequence alignment for both species revealed large numbers of alleles at multiple loci but overall diversity was lower than in the NSB. However, ratios of synonymous vs. non-synonymous substitutions in these sequences revealed little evidence of balancing selection, whereas in the NSB evidence for selection was strong. These data indicate that the strain of *C. psittaci* on Barrow Island has not driven the evolution of the observed MHC Class II B diversity. Furthermore, while the presence of an unknown pathogen may be a driver of evolution of the MHC Class II B in the NSB, another (possibly behavioural) mechanism is postulated as a more likely candidate to have produced the observed levels of sequence and locus diversity.

The studies reported in this thesis describe the discovery and characterisation of a panel of tetra-nucleotide microsatellite loci in the NSB genome utilising second-generation DNA sequencing. Population genetics analysis based on these loci has

shown deviations of all three populations from Hardy-Weinberg equilibrium and an associated degree of moderate inbreeding. Significant differentiation was also observed between the two translocated populations. DNA and amino acid sequence data and alignments from the Class II region of the NSB MHC were obtained. These data showed the presence of multiple Class II loci, each of which exhibited significant polymorphism. MHC Class II B in two other bird species was also high but failed to elucidate any relationship between pathogen load and sequence diversity. Drivers of MHC evolution in the NSB are therefore likely to be cryptic and possibly related to breeding behaviour.

The data generated in this study are the first to extensively characterise NSB at the genomic level. In particular the sequence data reported for the MHC Class II loci are unique and provide a basis for further characterisation of this important genomic region associated with adaptive immunity. Furthermore, these data may allow the augmentation of management strategies for the NSB, whilst improving our knowledge of the population dynamics and breeding system of this endangered bird species. Finally, this study lays foundations for developing a strategy for genetic management of NSBs and highlights key areas where knowledge is currently lacking, e.g. mating systems, pathogen loads in coexisting bird species and effective population size.

List of abbreviations

AMZ - Albany Management Zone

BA - Bald Island

BHI - Brain-heart Infusion

BLAST - Basic Local Alignment Search Tool

BSA - Bovine Serum Albumin

BWW - Black-and-white Fairy-wren

dN - Non-synonymous substitutions

dNTPs - Deoxynucleotide Triphosphates

DPaW - Western Austalian Department of Parks and Wildlife

dS - Synonymous substitutions

gDNA - Genomic DNA

H_2O - Water

H_E - Expected Heterozygosity

H_O - Observed Heterozygosity

HLA - Human Leucocyte Antigen

HWE - Hardy-Weinberg Equilibrium

IPTG - Isopropyl β-D-1-thiogalactopyranoside

IUCN - International Union for the Conservation of Nature

JTT - Jones-Taylor-Thornton model

LB - Luria broth

LD - Linkage Disequilibrium

LOXL4 - lysyl oxidase 4

MAL - Mean Alleles per Locus

MAR - Mean Allelic Richness

MCMC - Markov-Chain Monte-Carlo

MHC - Major Histocompatibility Complex

MG - Mt Gardner

$MgCl_2$ - Magnesium Chloride

ML - Maximum Likelihood

MP - Maximum Parsimony

MP - Mt Manypeaks

Mt - Mount

N_e - Effective Population Size

SGS - Second-generation Sequencing

NJ - Neighbour-Joining

NSB - Noisy Scrub-bird

PBR - Peptide Binding Region

PBS - Phosphate Buffer Solution

PCR - Polymerase Chain Reaction

QTL - Quantitative Trait Locus

SE - Standard Error

SLS - Sample Loading Solution

SNP - Single-Nucleotide Polymorphism

SPB - Spinifexbird

TAE - Tris-acetate EDTA buffer

TLR - Toll-like receptor

T_m - Optimal melting temperature

TPB - Two Peoples Bay

UV - Ultra-violet light

WA - Western Australia

X-Gal - 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

Chapter 1 – Background

1.1 Threats to global biodiversity

At present 20,000 species are listed as being threatened with extinction (IUCN, 2012) and currently the rate of extinction is 100 to 1000 times pre-human levels as indicated by the fossil record (Pimm *et al.*, 1995), leading some scientists to suggest we are facing a 'mass extinction' equivalent to the five previous mass extinctions over the last 540 million years or more (Barnosky *et al.*, 2011). The last mass extinction occurred 65 million years ago at the end of the Cretaceous Period and led to the extinction of the dinosaurs and the next event could be upon us within 240 years (Hooper *et al.*, 2012). Meanwhile, there is currently more global diversity in organisms as well as "their genetic and phenotypic variation and the ecosystems and communities in which they are a part" (Dirzo & Raven, 2003) (otherwise referred to as 'biodiversity') than at any other period in the earth's history (Rosenzweig, 1995). Biodiversity is understood to have a key role in ecosystem function (Balvanera *et al.*, 2006) and loss of biodiversity may have a detrimental impact on the services provided to mankind by ecosystems (Myers, 1997; Cardinale *et al.*, 2012; Pereira *et al.*, 2012). This includes the genetic component of biodiversity (Frankel, 1974; Myers, 1997). Threats to biodiversity are myriad but most are directly or indirectly anthropogenic, from the divisive issue of climate change to habitat loss, invasive species introduced by humans to direct persecution either for food or through competition for resources (Spangenberg, 2007; Rodríguez-Labajos *et al.*, 2009; Pereira *et al.*, 2012). These threatening processes have led to declines and extinctions throughout the entire eukaryote taxa.

The need to conserve biodiversity may be considered by some to represent a philosophical paradigm, in which proponents and sceptics argue whether the current rate of extinction of organisms represents an inexorable and terminal decline in the health of planet Earth or rather a natural process of attrition that is driven by the dynamic character of natural ecosystems. The latter argument is attractive, since it implies that extinction can be excused or even embraced as the logical end-point of the process of evolution by natural selection. The idea that the 'survival of the fittest' dooms those species that are unable to adapt to changes in their environment to extinction is a popular one that is hard to contest, especially as the ebb and flow of different forms of organisms is as old a process as life itself.

However, the perpetuation of the belief that extinction is an unfortunate but necessary by-product of the often brutal process of evolution is a misconception that needs to be addressed in the first instance. As previously stated, modern extinction rates are several orders of magnitude higher than prior to the appearance of humans (Pimm *et al*., 1995; Balvanera *et al.*, 2006). Previously the rate of extinction could be expected to be equalled or even outstripped by the emergence of new forms, especially as there are now more species than any other time in the Earth's history (Rosenzweig, 1995). So, while the gradual attrition of species is undeniably part of the evolutionary dynamic, the current rate of attrition is without precedent in the last 65 million years (Hooper *et al.*, 2012). This is unequivocally significant and such a rapid loss of biodiversity can be expected to have some detrimental impact on the planet.

Biodiversity does not simply represent species as single entities but rather encompasses the enumerable phenotypic and genetic variation found within whole communities and ecosystems (Dirzo & Raven, 2003). Therefore, the importance of conserving biodiversity goes beyond the value (monetary or otherwise) applied to individual or even groups of species. The phrase 'biodiversity-ecosystem services' represents the benefits that biodiversity and ecosystems provide to human and provides a quantifiable measure of the value of biodiversity. Examples of biodiversity-ecosystem services include the pollination of crops, carbon sequestration and biocontrol of pests and, although linking the role of biodiversity in augmenting these services is challenging, there is a growing body of evidence that shows that this is the case for many biodiversity-ecosystem services (Balvanera *et al.*, 2006; Duffy, 2009; Cardinale *et al.*, 2012). As an example of the value of these services, Costanza *et al.* (1997) put their global economic value at US$33 trillion (US$44 trillion today (Holzman, 2012)), nearly twice global Gross National Product.

However, there is also an argument that asserts that the intrinsic value of biodiversity goes beyond simple economics to something eminently less tangible. Wilson (1984) coined the term 'Biophilia' as the "innate tendency to focus on life and life-like beings", which in essence is the fascination that humans have with the natural world. Biophilia's grasp on the human psyche can be seen throughout history and pre-history from cave-paintings to friezes, frescoes and murals to the symbolism of animals on totems, coats-of-arms, flags and even sports teams. Humanity's bond with (and the cultural significance of) biodiversity is undeniable and the impetus to

conserve species and stall the current crescendo of extinctions is not merely a philosophical paradigm, but rather an ecological, economic and cultural imperative.

1.2 Biodiversity conservation and genetics

1.2.1 Genetic diversity as biodiversity

The need to conserve biodiversity at a genetic level is also highly important (Frankel, 1974; Frankham, 2005), although it is a facet of biodiversity that we least understand (Pereira *et al.*, 2012). As populations of species decline towards extinction, they experience a loss of the genetic variability available through the processes of genetic drift and inbreeding (Keller & Waller, 2002). A reduction in this genetic diversity can negatively effect populations, either through the increased likelihood of recombination of deleterious alleles (which may directly reduce fitness) or simply by reducing the evolutionary potential of a population, i.e. the ability of a population to withstand and adapt to stochastic changes to their environment. Therefore, the genetic component of biodiversity can be important for the short-term survival of extant taxa, or it may represent a significant part of the evolutionary heritage of a species which may in turn influence the future evolution of its descendants.

Genetic diversity in a conservation context can be quantified in different ways, according to the type of genetic markers used. These markers can be defined as either neutral or adaptive (functional) based on whether they occur in non-coding or coding regions of a genome, and therefore whether they are under selection constraints or not. Examples of the most commonly used markers are listed in Table 1.1. For example, microsatellites (single locus), minisatellites (multi-locus) and isozymes are among the most frequently used neutral markers and mitochondrial DNA (mtDNA) and Major Histocompatibility Complex (MHC) are commonly used adaptive markers. Neutral markers are general used to reflect global diversity across genomes, whereas adaptive markers quantify variation in specific functional gene groups. Some markers such as microsatellites and the MHC are highly polymorphic (Goldstein & Schlötterer, 1999; Hess & Edwards, 2002) and are popular markers for this reason. However, variation in adaptive markers is not directly linked to neutral variation (Holderegger *et al.*, 2006) and each must be examined in isolation.

Previous studies have shown that genetic diversity of populations or species is intrinsically linked to its extinction risk (Frankham, 2005; Evans & Sheldon, 2008)

and, therefore, the consideration of genetic factors should play a key role in the management of threatened populations. As a consequence, studies on genetic diversity in species of conservation concern are abundant in the scientific literature.

Work with the cheetah (*Acinonyx jubatus*) has underlined the importance of genetic studies in understanding how low genetic variability within populations can have detrimental impacts on fitness (O'Brien *et al.*, 1983; O'Brien *et al.*, 1985; O'Brien *et al.*, 1987). It appears that cheetahs have undergone at least two major population bottlenecks and are the most genetically depauperate among felid species (O'Brien *et al.*, 1987). Captive populations were found experience significantly reduced fecundity (semen quality) (Wildt *et al.*, 1983) as well as fluctuating assymmetry of their skeletal form (Wayne *et al.*, 1986). Furthermore, allogenic skin grafts between unrelated individuals were accepted and this was possible due to a monomorphism at the Major Histocompatibility Complex (MHC) locus. However, a later study found that the immunocompetence of wild cheetahs has not be impacted by low MHC diversity, reflecting a paradox which may relate to the difference in conditions experienced by captive and wild populations (Castro-Prieto *et al.*, 2011). This study compares the low MHC diversity in cheetah populations and similarly low diversity in other mammals, such as the Northern elephant seal (*Mirounga angustirostris*) which have no apparent influence on immunocompetence (Weber *et al.*, 2004), with the desert bighorn sheep (*Ovis aries*) which has high MHC diversity but a high susceptibility to a number of diseases (Gutierrez-Espeleta *et al.*, 2001) . Clearly, it is of high importance to put the results of genetic analysis into the both context of the populations being investigated and the results of other similar studies. Furthermore, other work has found that despite low MHC variation, Cheetahs retain extensive microsatellite diversity (Menotti-Raymond & O'Brien, 1995) highlighting the importance of using both adaptive and neutral markers to ascertain an informed assessment of genetic diversity in a taxon.

However, another example of how low genetic diversity in a population can be highly detrimental to fitness is the Florida subspecies of puma, also known as the Florida panther (*Puma concolor coryi*). It is estimated that this population went through a bottleneck as low as six individuals (Culver *et al.*, 2008) and now it only numbers 70-80 after genetic restoration with puma from Texas (Johnson *et al.*, 2010; IUCN, 2012). Two morphological characteristics of the low genetic diversity resulting from this bottleneck are apparently harmless (cow-lick & tail-kink).

Table 1.1 Examples of bird taxa of conservation significance that have been subjects of genetic diversity studies

Species	Scientific Name	Bottleneck	IUCN listing*	Trend*	Study Markers	References
Asian crested ibis	*Nipponia nippon*	7 & 5†	EN	increasing	microsatellites, MHCα	He *et al.*, 2006; Zhang *et al.*, 2006
black robin	*Petroica traversi*	5	EN	increasing	minisatellites, MHCα	Ardern & Lambert, 1997; Miller & Lambert, 2004a
California condor	*Gymnogyps californianus*	14	CR	increasing	mtDNA∞, minisatellites, microsatellites	Geyer *et al.*, 1993; Adams & Villablanca, 2007; Romanov *et al.*, 2009
Forbes' parakeet	*Cyanoramphus forbesi*		EN	Increasing	mtDNA, microsatellites	Boon *et al.*, 1999; Chan *et al.*, 2006; Chan *et al.*, 2009
Galápagos hawk	*Buteo galapagoensis*	n/a	VU	stable	minisatellites, microsatellites, MHCα	Bollmer *et al.*, 2005; Bollmer *et al.*, 2011
golden-cheeked warbler	*Dendroica chrysoparia*	n/a	EN	decreasing	microsatellites	Lindsay *et al.*, 2008
great Indian bustard	*Ardeotis nigriceps*	n/a‡	CR	decreasing	mtDNA∞	Ishtiaq *et al.*, 2011
greater prairie-chicken	*Tympanuchus cupido*	n/a	VU	decreasing	mtDNA∞, microsatellites, MHCα	Johnson *et al.*, 2003; Eimes *et al.*, 2010
Hawaiian goose	*Branta sandvicensis*	c.30	VU	increasing	minisatellites, microsatellites	Rave, 1995; Veillet *et al.*, 2008
Laysan finch	*Telespiza cantans*	c.100	VU	stable	microsatellites	Tarr *et al.*, 1998
little spotted kiwi	*Apteryx owenii*	5§	NT	stable	microsatellite, MHCα	Ramstad *et al.*, 2010; Miller *et al.*, 2011
Madagascar fish-eagle	*Haliaeetus vociferoides*	n/a‡	CR	decreasing	microsatellites	Johnson *et al.*, 2009
Mariana crow	*Corvus kubaryi*	n/a‡	CR	decreasing	mtDNA∞, minisatellites, microsatellites	Tarr & Fleischer, 1999
Mauritius kestrel	*Falco punctatus*	4	VU	decreasing	microsatellites	Groombridge *et al.*, 2000
millerbird	*Acrocephalus familiaris*	<50	CR	stable	mtDNA∞, microsatellites	Addison & Diamond, 2011
Okinawa rail	*Gallirallus okinawae*	717	EN	decreasing	mtDNA∞	Ozaki *et al.*, 2010
Rarotonga monarch	*Pomarea dimidiata*	29	VU	stable	mtDNA∞; microsatellites	Chan *et al.*, 2011
saddleback	*Philesturnus carunculatus*	36¤	NT	increasing	microsatellites, isozyme	Lambert *et al.*, 2005; Taylor *et al.*, 2007
Seychelles warbler	*Acrocephalus sechellensis*	<30	VU	increasing	microsatellites, MHCα	Richardson *et al.*, 2000; Richardson & Westerdahl, 2003
Spanish imperial eagle	*Aquila adalberti*	30 pairs	VU	increasing	mtDNA∞; microsatellites	Martinez-Cruz *et al.*, 2004
stitchbird	*Notiomystis cincta*	n/a	VU	stable	microsatellites	Brekke *et al.*, 2011
whooping crane	*Grus americana*	14	EN	increasing	microsatellites, mtDNA∞	Glenn *et al.*, 1997; Glenn *et al.*, 1999

(Order Passeriformes in **bold)** *IUCN listing and population trend from IUCN (2012) IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 18 November 2012, CR – Critically Endangered, EN – Endangered, VU – Vulnerable, NT – Near Threatened; † figures for separate bottlenecks in Chinese and Japanese populations respectively (BirdLife International 2012. *Nipponia nippon.* In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 18 November 2012; ‡ bottleneck likely still ongoing based on current population trends; § figure represents bottleneck on Kapiti Island, from which other island populations are mostly descended from (Miller *et al.*, 2011); ¤ figure represents South Island subspecies *Philesturnus carunculatus carunculatus* (Taylor *et al.*, 2007); α Major Histocompatibility Complex; ∞ mitochondrial DNA

However, other characteristics are more clearly fitness-related, such as high incidences of abnormal sperm-morphology, poor seminal quality, cryptorchidism (or undescended testes), cardiac defects (atrial septum) and high pathogen loads (Roelke *et al.*, 1993).

These examples show the impact that low genetic diversity can potentially have and underlines the importance of retaining as much of the genetic component of biodiversity as possible. There is an extensive range of other examples of genetic studies of species of conservation interest that have undergone population bottlenecks or long-term declines. Table 1.1 shows some of the work that has been done on a broad range of bird taxa, encompassing species endemic to single islands or archipelagoes (e.g. black robin, Galápagos hawk, Laysan finch etc), species with historically small distributions (e.g. golden-cheeked warbler, Spanish imperial eagle) as well as continental species whose distributions were previously much larger (e.g. whooping crane, greater prairie-chicken). However, all these species are of current conservation concern, as denoted by their listing on the International Union for the Conservation of Nature's (IUCN) Red List. While none of the species listed are at the highest priority level (Extinct in the Wild), five species are listed at the second highest tier, Critically Endangered, three of which (Madagascar fish-eagle, great Indian bustard, Marina crow) are currently understood to be still in decline (IUCN, 2012) and therefore currently still experiencing a population bottleneck. Other species have recovered from bottlenecks as small as 4-5 wild individuals to as large as 717 in the case of the Okinawa rail. For some other species the loss of diversity has occurred across multiple populations, where the size of specific bottlenecks are hard to ascertain. However, the uniting factor in all of these avian genetics studies is relatively low genetic diversity in post-bottleneck populations.

Unlike the cheetah and the Florida panther, the impact of low genetic diversity on fitness or the ability of many of these species to recover is not clear. However, there are several studies that have found strong links between genetic diversity and fitness-related traits in birds, such as pathogen resistance (Worley *et al.*, 2010), immunocompetence (Tompkins *et al.*, 2006; Hale & Briskie, 2007) and fecundity (Briskie & Mackintosh, 2004; Ortego *et al.*, 2007a), although other studies have shown that this relationship may not always be the case (Ortego *et al.*, 2007b). Nevertheless, the weight of evidence suggests that a reduction in genetic diversity is

likely to affect the ability of threatened species to recover and adapt to new environmental challenges.

As already mentioned, it is clear that low genetic diversity is inherent tied to increased extinction risk. However, unlike the cheetah and the Florida panther, the impact of low genetic diversity on fitness or the ability of many of these species to recover is not clear. Nevertheless, there are several studies that have found strong links between genetic diversity and fitness-related traits in birds, and while these studies have focused on more widespread species, they present evidence that low genetic diversity and inbreeding can have a significant impact. For example, Worley *et al.* (2010) found that red junglefowl (*Gallus gallus*) that are heterozygous for the MHC are more likely to survive infection by coccidia parasites (i.e. 'heterozygote advantage'), with heterozygous surviving 42% longer on average than homozygotes. Furthermore, it was found that an inbred population of New Zealand robins (*Petroica australis*) showed a significantly reduced immune response to gastrointestinal parasites and a phytohaemagglutinin skin test than outbred individuals of the same species (Hale & Briskie, 2007). Fecundity in 22 species of native bird species from New Zealand was assessed through hatching failure and it was found that bottleneck size had a significant impact with average hatching failure 3.0 (±0.6%) in non-bottlenecked species compared to 25.3 (±5.0%) in species with bottleneckes under 150 individuals (Briskie & Mackintosh, 2004). Similarly, Ortego *et al.* (2007a) found that clutch size positively correlated with female heterozygosity in lesser kestrels (*Falco naumanni*). However, the same authors failed to find a similar relationship with avian malaria prevalence. Nevertheless, the weight of evidence suggests that a reduction in genetic diversity is likely to affect the ability of threatened species to recover and adapt to new environmental challenges.

There are examples of species in in Table 1.1 that have been used to demonstrate the relationship between genetic diversity and fitness and illustrates the need for conservation managers to be informed about the genetics of a threatened species. In the Galápagos hawk, a study of diversity in minisatellite loci (DNA-fingerprinting) found a strong negative correlation between genetic diversity and parasite loads and positive correlation between diversity and natural antibody levels (Whiteman *et al.*, 2006). The study found that inbred individuals had higher loads of parasites and lower immune responses compared to more outbred individuals and additionally the levels of antibodies in individuals was negatively correlated with the abundance of

lice, underlining the link between immune response and susceptibility to parasites. This study demonstrates clearly the effect that genetic diversity can have on fitness in this species and, as discussed in Whiteman *et al.* (2006), the information provided by this study will help inform management practices and strategies for the conservation of the Galápagos hawk. This is a key point as the rationale for many genetic diversity studies of conservation-dependent species is to improve and adjust the way we manage species threatened with extinction and the environments which they inhabit.

Another threatened bird species that has been shown to link low genetic diversity to fitness-related traits is the Forbes' parakeet (*Cyanoramphus forbesi*). This species was confined to two islands in the Chatham archipelago off New Zealand's South Island and one of these populations (Mangere Island) became extinct in 1930 and this island wasn't recolonised until 1973 (IUCN, 2012). The population on the remaining island (Little Mangere) was poorly known for this period. These populations have relatively low genetic diversity, compared to populations on the New Zealand mainland (Chan *et al.*, 2009). Tompkins *et al*. (2006) showed that immune function in this species is lower than in the closely related but outbred red-crowned parakeet (*Cyanoramphus novaezelandiae*). Interestingly, it was found that immune function in hybrids between the two species was also higher than in pure *C. forbesi* individuals, presenting a feasible rescue strategy for this taxon (Chan *et al.*, 2006; Tompkins *et al*., 2006).

Finally, the California condor has experienced a severe bottleneck of 14 individuals, which has led to an increased frequency in the allele for the lethal form of dwarfism known as chondrodystrophy (Ralls & Ballou, 2004). Of 169 eggs laid in captivity, five contained embryos which exhibited signs of chondrodystrophy and the frequency of this deleterious allele was estimated at 9% (Ralls *et al*., 2000). While captive breeding strategies have sought to mitigate this, the relatively high frequency of this lethal genetic mutation in the California condor further highlights the impact that low genetic diversity and inbreeding can have on threatened bird species.

In summary, there is a strong body of evidence showing the important of genetic diversity and associated inbreeding depression in influencing extinction risk through impacts on fitness. This includes a range of bird species, with some empirical evidence coming from those species that are most at risk of becoming extinct, including species restricted to island populations.

Table 1.2 Examples of island bird taxa of conservation significance that have had their genetic diversity evaluated

Species	Scientific Name	Bottleneck Size	IUCN Status*	Population Trend*	Reference(s)
Abbott's booby	*Papasula abbotti*	n/a	EN	decreasing	Morris-Pocock *et al.*, 2012
Amsterdam albatross	*Diomedea amsterdamensis*	5 pairs	CR	decreasing	Milot *et al.*, 2007
Balearic shearwater	*Puffinus mauretanicus*	n/a	CR	decreasing	Genovart *et al.*, 2012
black robin	*Petroica traversi*	5	EN	increasing	Ardern & Lambert, 1997; Miller & Lambert, 2004a
Chatham Island taiko	*Pterodroma magentae*	> 4 pairs	CR	increasing	Lawrence *et al.*, 2008
Christmas Island frigatebird	*Fregata andrewsi*	n/a	CR	decreasing	Morris-Pocock *et al.*, 2012
Forbes' parakeet	*Cyanoramphus forbesi*		EN	increasing	Boon *et al.*, 1999; Chan *et* al., 2006; Chan *et al.*, 2009
Galápagos hawk	*Buteo galapagoensis*	n/a	VU	stable	Bollmer *et al.*, 2005, Bollmer *et al.*, 2011
Guam rail	*Gallirallus owstoni*	22	EW	n/a	Haig & Ballou, 1995
Hawaiian goose (nene)	*Branta sandvicensis*	c.30	VU	increasing	Rave, 1995; Veillet *et al.*, 2008
kakapo	*Strigops habroptilus*	<50	CR	increasing	Miller *et al.*, 2003
Laysan finch	*Telespiza cantans*	100	VU	stable	Tarr *et al.*, 1998
little spotted kiwi	*Apteryx owenii*	5	NT	stable	Ramstad *et al.*, 2010; Miller *et al.*, 2011
Mariana crow	*Corvus kubaryi*	n/a	CR	decreasing	Tarr & Fleischer, 1999
Mauritius kestrel	*Falco punctatus*	4	VU	decreasing	Groombridge *et al.*, 2000
Mauritius parakeet	*Psittacula eques*	>10	EN	increasing	Raisin *et al.*, 2009
millerbird	*Acrocephalus familiaris*	<50	CR	stable	Addison & Diamond, 2011
Okinawa rail	*Gallirallus okinawae*	717	EN	decreasing	Ozaki *et al.*, 2010
palila	*Loxioides bailleui*	n/a	CR	decreasing	Fleischer *et al.*, 1994
pink pigeon	*Nesoenas mayeri*	10	EN	decreasing	Swinnerton *et al.*, 2004
Puerto Rican amazon	*Amazona vittata*	13	CR	stable	Beissinger *et al.*, 2008
Rarotonga monarch (kakerori)	*Pomarea dimidiata*	29	VU	stable	Chan *et al.*, 2011
saddleback	*Philesturnus carunculatus*	36	NT	increasing	Lambert *et al.*, 2005; Taylor *et al.*, 2007
Seychelles warbler	*Acrocephalus sechellensis*	<30	VU	increasing	Richardson & Westerdahl, 2003; Brouwer *et al.*2007
stitchbird (hihi)	*Notiomystis cincta*	n/a	VU	stable	Brekke *et al.*, 2011
takahe	*Porphyrio hochstetteri*	250-300	EN	increasing	Grueber & Jamieson, 2011

*IUCN listing and population trend from IUCN (2012) IUCN Red List of Threatened Species. Version 2012.2. <www.iucnredlist.org>. Downloaded on 16 December 2012, EW – Extinct in the Wild, CR – Critically Endangered, EN – Endangered, VU – Vulnerable, n/a - data not available or no bottleneck applicable to this species (i.e. population decreasing).

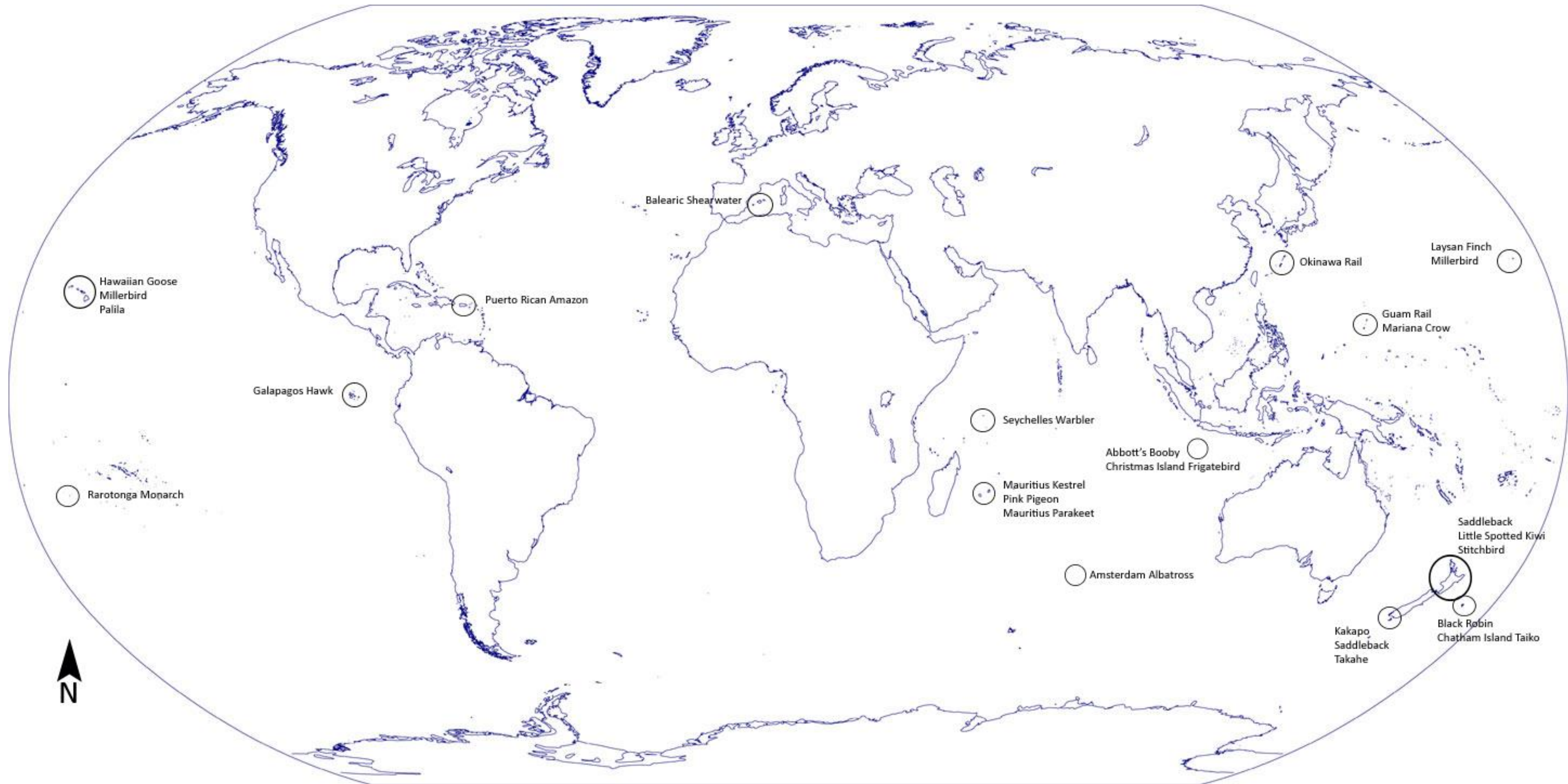


Figure 1.1 World map showing geographic location of islands/archipelagoes where threatened bird species currently/previously occurred (adapted from Nations Online Project (http://www.nationsonline.org))

1.2.2 Conserving genetic diversity in island bird species

The conservation of endemic species on islands presents some unique challenges, not least that island populations tend to be naturally small. The taxonomic group that best illustrates the challenges that island species face, and how that impacts on genetic diversity, are birds. Figure 1.1 shows the geographical distribution of some of the examples of island bird species of conservation significance that have had their genetic diversity evaluated. These species cover a broad range of avian forms and taxa, including pelagic seabirds that return to specific islands to breed (Abbott's booby, Amsterdam albatross, Balearic shearwater, Chatham Island taiko, Christmas Island frigatebird) to sedentary species including several that are completely flightless (Guam rail, kakapo, little spotted kiwi, Okinawa rail, takahe). Table 1.2 shows the size of the bottlenecks experienced by these species and their current population trends. While some of these species show remarkably high genetic diversity given their population histories (e.g. Chatham Island taiko (Lawrence *et al.*, 2008); stitchbird (Brekke *et al.*, 2011), generally most island species that have been studied have relatively low genetic diversity.

MacArthur and Wilson (1967) were among the first to understand that ecological processes on islands are different from those in mainland systems and coined the term 'island biogeography' or 'island theory'. One of the key principles of island biogeography is that extinction rates are intrinsically higher on smaller islands than large ones and this has been reinforced in other work (Diamond, 1984; Vitousek, 1988; Flesness, 1989; Case *et al.*, 1992; World Conservation Monitoring Centre, 1992; Smith *et al.*, 1993). Furthermore, we understand that small isolated populations are more vulnerable to stochastic environment and demographic pressures, which can elevate extinction risk (Keller & Waller, 2002). Therefore, theoretically island species limited to one or a few small islands will be at greater extinction risk than species found across a large island or archipelago, which in turn will be at less risk than continental species.

This theory is validated by the fact that the majority of extinctions in the last 400 years have been of island species (Frankham, 1997). The extinctions of birds in particular have mainly been on islands (Johnson & Stattersfield, 1990; Donald *et al.*, 2010) with 39% of all threatened bird species being on islands (Johnson & Stattersfield, 1990) despite the fact only 20% of all birds are found on islands

(Diamond *et al.*, 1989). Therefore, the species in Table 1.2 represent a key theme in species conservation, in that there is a strong emphasis on the threat to island species. Traditionally, the main risk factors for island extinctions have been those tangible threats that are observeable and quantifiable. For example, non-native invasive species introduced (usually by humans) to islands have been identified as past or present threats through competition or predation for 100% of the island species listed in Table 1.2 (IUCN, 2012). This is unlikely to be coincidental and the introduction of non-native species by humans is often ranked as the top threat to island endemics. This is a view shared by Blackburn *et al.* (2004) and Duncan & Blackburn (2004) who show positive correlations with the introduction of alien mammalian predators and extinction probability. However, mammalian predators are only part of the story, since e.g. yellow crazy ants (*Anoplolepis gracilipes*), guava (*Psidium cattleianum*) and brown tree-snake (*Boiga irregularis*) are, or have been, significant threats for Abbott's booby, Mauritius parakeet and Guam rail respectively (IUCN, 2012). Furthermore, threats such as habitat loss/fragmentation (e.g. Christmas Island frigatebird; Puerto Rican amazon), disease (Hawaiian goose; pink pigeon; Hawaiian honeycreepers (Drepanidinae) (which includes palila & Laysan finch) (Jarvi *et al.*, 2001)) and continued pressure from hunting and persecution (Galápagos hawk) (IUCN, 2012) have been implicated in the declines of many other threatened bird species.

However, Jamieson (2007) argues that the threat that non-native species pose to island ecosystems should not be viewed in isolation and that genetic diversity and inbreeding both have a key role in extinction risk. Frankham (1997) presented empirical evidence of intraspecific genetic variation (allozyme) in island and mainland populations from a wide range of taxa and found that in 165 of 202 comparisons, variation was lower on islands. Furthermore, in 34 of 38 endemic island species allozyme variation was lower than in closely related mainland species. This evidence was followed up by Frankham (1998) who found that inbreeding coefficients were significantly higher in island populations, especially in endemic island species. Ardern & Lambert (1997) found that much of the low minisatellite diversity in the black robin was due to its persistence on one small island for ~100 years, rather than the extreme bottleneck of five birds (Table 1.1) (including just one breeding female) c.1980.

As already discussed, genetic diversity is intrinsically linked to extinction risk (Brook *et al.*, 2002; Evans & Sheldon, 2008; Frankham, 1996; Frankham, 2005) and reduced fitness (Keller & Waller, 2002; Rolke *et al.*, 1993), so in light of this evidence it must be accepted that island populations must have a inherently higher extinction risk compared to mainland populations. Accordingly, genetic factors are listed as threats for many island bird species (e.g. Galápagos hawk; Hawaiian goose; millerbird etc (IUCN, 2012), reflecting their perceived importance.

Nevertheless, given the large body of evidence that the primary risk factors in island exinctions are environmental it seems a reasonable assertion that genetic factors work in tandem with other threats (e.g. mammalian predators) to increase extincton risk rather than isolation (Duncan & Blackburn, 2004; Reed, 2007). However, this doesn't account for the fact that low genetic diversity and high inbreeding coefficients appear to be intrinsic to island populations. Therefore, island populations are inherently vulnerable, not just through biogeography but also genetically, to environmental stochasticity, including (but not limited to) incursions by invasive species. The overall extinction risk presented to island populations by invasive species, climate change, novel pathogens etc, is secondary to their fundamental genetic vulnerability.

Figure 1.1 shows a global distribution of selected island species. These species are, or have been, of such significant conservation concern that researchers and conservation managers have felt it necessary to gain a better understanding of conservation genetics. While these species are widespread across three different oceans, three specific archipelagoes stand out as being of particular interest: New Zealand (and surrounding islands e.g. Chatham Islands), Hawai'i (including outlying islands e.g. Laysan and Nihoa) and Mauritius. The original pre-European colonisations of New Zealand and Hawai'i precipitated extinctions of many species in the bird-rich biota of these islands (Diamond *et al.*, 1989; Pimm *et al.*, 1994; Duncan & Blackburn, 2004), not least the large, flightless species such as the moa (Dinornithidae) in New Zealand and the goose-like moa-nalo (*Thambetochen spp.*) in Hawai'i, for which hunting for food was a major contributing factor (Duncan *et al.*, 2002; Donald *et al.*, 2010; Hume & Walters, 2012)[1]. However, the arrival of Europeans to these islands has initiated a lengthy attrition of native species, with

[1] On Mauritius, hunting by European settlers was also implicated in the extinction of the flightless dodo (*Raphus cucullatus*) but this was unlikely to be the primary reason (Hume & Walters, 2012).

Hawai'i losing 34% of its endemic birds and the Mascarene Islands (including the islands of Mauritius, Réunion and Rodrigues) losing 50% of its native bird fauna (Johnson & Stattersfield, 1990) since the European colonisation. Some of these extinctions have continued up until relatively recently, with the disappearance of the Kauai o'o (*Moho braccatus*) in the Hawaiian archipelago after 1987 and the bush wren (*Xenicus longipes*) in New Zealand since 1972 (IUCN, 2012). Fortunately, conservation efforts have been successful in preventing the loss of more species, such as those listed in Table 1.2 but, as previously discussed, the impact of the threatening processes (non-native species introductions, habitat loss and fragmentation, hunting etc) has had an impact on the genetics of some island species, which may already be vulnerable due to intrinsically low genetic diversity (Frankham, 1998).

Indeed, the declines and near-extinctions of e.g. black robin, kakapo, saddleback, takahe and little spotted kiwi in New Zealand, Hawaiian goose, millerbird, Laysan finch and palila in Hawai'i and pink pigeon, Mauritius kestrel and Mauritius parakeet in Mauritius appear to have led to generally low genetic diversity in these species. The black robin, for example, was reduced to just one breeding female and consequently has been shown to have lost diversity at neutral markers (Ardern & Lambert, 1997) and, more significantly, is monomorphic for Major Histocompatibility Complex Class II B, an important immunological gene (Miller & Lambert, 2004a). A pre- and post-bottleneck study of the Mauritius kestrel has shown a dramatic 55% reduction in allelic diversity (Groombridge *et al.*, 2000) and the extinction of the South Island subspecies of saddleback from the mainland led to a reduction from 143 to 35 alleles at 22 loci (Taylor *et al*. 2007). An analysis of the genetic diversity of the extant Nihoa subspecies of millerbird (*Acrocephalus familiaris kingi*) found extremely low variability in both microsatellite and mitochondrial markers, with 10 of 14 microsatellite loci being fixed at one allele (Fleischer *et al.*, 2007), probably as a result of multiple bottlenecks (Conant & Morin, 2001). The only apparent exception is the stitchbird, which appears to have retained surprising levels of genetic diversity, in contrast to other threatened birds in New Zealand (Brekke *et al.*, 2011) and indeed most other island species worldwide.

All of species dealt with thus far have been endemic to true islands or archipelagoes. However, 'islands' of habitat can occur in mainland ecosystems (MacArthur & Wilson, 1967) and corridors that may previously have provided a degree of

connectivity between ‘islands’ can be eroded, leading to increased fragmentation (Wiens, 1995). Species that are restricted to these pockets of habitat may then be geographically isolated in the same way as island species are, leading to a reduction in genetic diversity through genetic drift or inbreeding or a combination (Frankham, 1997; Frankham, 1998; Keller & Waller, 2002). In this study, I maintain that the one area of isolated habitat at Two Peoples Bay Nature Reserve, which the distribution of the noisy scrub-bird contracted to, represents the equivalent of an island with all the associated challenges that island populations may face.

1.3 Translocations and biodiversity conservation

1.3.1 Translocations as conservation management strategy

Translocations have become a useful and popular tool in the conservation of populations of threatened species (Griffith *et al.*, 1989; Pullin, 2002; Rout *et al.*, 2007), and generally entail the introduction of individuals from one population into a) a location where the species was previously extant (reintroduction) (Armstrong & Seddon, 2008) b) a location where the species is not believed to have been extant but where suitable habitat exists (introduction) or c) a location where the species is currently extant to ameliorate the gene pool at that location (genetic restoration) (Hedrick, 2005; Bouzat *et al.*, 2009). The primary aim of most translocations is to reduce extinction risk of a species through establishing new populations, thereby minimising the likelihood that a stochastic event will result in the loss of the entire species. To date, translocations have been executed successfully for a wide range of taxa, for example the IUCN Reintroduction Specialist Group (RSG) has published reports of successful translocations of plants (e.g. Corrigin grevillea (*Grevillea scapigera*) (Dixon & Krauss, 2008) and *Banksia montana* in south-western Western Austalia (Barrett *et al.*, 2011)), invertebrates (e.g. leaf-vein slug (*Pseudaneitea maculata*) (Bowie, 2010a) and Banks Peninsula tree weta (*Hemideina ricta*) (Bowie, 2010b) in New Zealand), fish (e.g. Yarqon bleak (*Acanthobrama telavivensis*) in Israel (Goren, 2010) & Colorado pikeminnow (*Ptychocheilus lucius*) in the western USA (Bain, 2010)), amphibians (e.g. natterjack toad (*Epidalia* (*Bufo*) *calamita*) (Griffiths *et al.*, 2010) and Romer’s tree-frog (*Chirixalus (Philautus) romeri*) in Hong Kong (Lau & Banks, 2008)), reptiles (e.g. shore skink (*Oligosoma smithi*) (Baling *et al.*, 2010) in New Zealand and Antiguan racer (*Alsophis antiguae*) (Daltry

et al., 2010), birds (e.g. noisy scrub-bird (*Atrichornis clamosus*) (Comer *et al.*, 2010) in south-western Western Australia and red kites (*Milvus milvus*) in the UK (Murn *et al.*, 2008)), and mammals (e.g. Arabian oryx (*Oryx leucoryx*) in Saudi Arabia and United Arab Emirates (Kiwan *et al.*, 2008; Simkins, 2008; Zafar-ul Islam *et al.*, 2010) and grey wolves (*Canis lupus*) in Wyoming and Idaho, USA (Bangs & Smith, 2008).

However, while many translocations have proved successful in reducing extinction risk, there may be an array of potential issues to overcome in order to achieve this. A clear understanding of the ecology of the species and associated taxa is an important prerequisite as potential translocation sites must be assessed for suitability and the impact on existing taxa must be understood and potentially ameliorated. Translocation strategy must also be adaptive to allow for issues that may be apparent later. For example, when threatened species populations are subjected to threats from alien predators, there is some evidence that individuals translocated from source populations where predators are not present (e.g. zoos) may be naïve and more vulnerable to predation (Whitwell *et al.*, 2012). In these cases, predator awareness training has often proved successful.

The use of islands is a common feature in many translocations and New Zealand presents a particularly good example in this regard. The translocation of six species of bird (kakapo (Miller *et al.*, 2003), little spotted kiwi (Miller *et al.*, 2011), saddleback (Taylor & Jamieson, 2008), black robin (Ardern & Lambert, 1997), stitchbird (Brekke *et al.*, 2011), takahe (Grueber & Jamieson, 2011)) native to New Zealand (Table 1.2 and Figure 1.1) to islands (both offshore within lakes as well as habitat 'islands' (Jamieson, 2009)) has been instrumental in the successful recovery of these species (Jones & Merton, 2012). This has largely been to alleviate extinction risk quickly by removing populations from threats (e.g. non-native predators) (Jamieson, 2009) initially, while methods of controlling those threats are put into place. Additionally, a translocation program has established new populations of Laysan finch (Tarr *et al.*, 1998) and another is underway to translocate Nihoa millerbird (subspecies *kingi*) to Laysan Island where another subspecies (*familiaris*) formerly occurred (Farmer *et al.*, 2011). Islands have also been used in the translocations of other taxa other than birds. In New Zealand, for example, robust (*Oligosoma alani*), Suter's (*O. suteri*) and Whitaker's skinks (*O. whitakeri*) and Auckland tree weta (*Hemideina thoracica*) have all been successfully translocated to

Korapuki Island in the Mercury Islands; the weevil *Hadramphus stilbocarpae* was successfully introduced to Breaksea Island; Cook Strait giant weta (*Deinacrida rugosa*) to Maud and Maitu/Somes Islands; Mercury Island tusked weta (*Motuweta isolata*) to Red Mercury Island (all Sherley *et al.*, 2010). And, although translocation attempts of lesser short-tailed bats (*Mystacina tuberculata*) to Kapiti and Ulva Islands were not considered to be successful (Sherley *et al.*, 2010), strategies were revised and met with more success with a follow-up release on Kapiti (Ruffell & Parsons, 2010). Finally, the translocations of noisy scrub-birds and Gilbert's potoroos to Bald Island in south-west Western Australia have both proved successful (Comer *et al.*, 2010; Garnett *et al.*, 2011; Roache, 2011) as have translocations of Barrow Island golden bandicoot (*Isoodon auratus barrowensis*), white-winged fairy-wrens (*Malurus leucopterus edouardi*) and spinifexbirds (*Eremiornis carteri*) to Hermite Island in the Montebello Group (A.H. Burbidge *pers. comm.*).

1.3.2 Translocations and genetic diversity

Translocations (particularly those involving islands) have a key role to play in the conservation of threatened species. However, an important factor that has often not been well addressed in translocations are genetic considerations. In the case of local or co-adaptation, the hybridisation of individuals sourced from populations in differerent environments may lead to a loss of fitness, i.e. outbreeding depression (Templeton *et al.*, 1986). Additionally, maladaptation to environments may be equally detrimental to the success of a translocation (Tufto, 2001). Moreover, the genetic variation available in the parent and founder populations is also an important consideration and one which will be a significant aspect of this thesis.

Translocations may use individuals from existing wild populations or from captive-breeding (Sarrazin & Barbault, 1996) and the source and number of translocation founders will have an impact on the genetics of the new population (Armstrong & Seddon, 2008). However, the source and size of a translocation will not only have an inherent impact on the genetics of the translocated (secondary) population but also potentially on the parent (primary) population (Armstrong & Seddon, 2008; Bain & French, 2009). Translocations can also be used as a tool to restore genetic diversity to an existing population and, while the effectiveness of genetic 'rescue' or 'restoration' can be complex (Tallmon *et al.*, 2004), in theory (Hedrick, 1995; Ingvarsson, 2001; Hedrick 2005) and also in practice (Bouzat *et al.*, 2009; Johnson *et*

al., 2010) it remains a potentially useful conservation tool for reducing the genetic 'load' in a population. Genetic load is the loss of fitness through the build up of deleterious alleles through the processes of mutation, segregation etc (Crow & Kimura, 1970) and in small populations can lead to inbreeding depression (Keller & Waller, 2002), which we already know positively correlates with extinction risk of a population. It has already proved useful in alleviating inbreeding depression in Florida panthers (Johnson *et al.*, 2010) and also greater prairie-chickens (Bouzat *et al.*, 2009) and could potentially be of use in ameliorating low genetic diversity and genetic load in other species (e.g. stitchbird (Brekke *et al.*, 2011)). In fact, Heber & Briskie (2013) showed that reciprocal translocations of New Zealand robins as part of a genetic restoration could result in reduced inbreeding depression (i.e. increased fitness), even if the populations involved were inbred and translocation numbers were small. This example is striking in that it suggests that even if the only donor populations available are themselves inbred that there is still merit in undertaking a for a genetic rescue/restoration. However, when undertaking genetic rescues, as with any articifical mixing of genetic populations, consideration should always be given to the possibility of outbreeding depression (see above).

One of the main impacts of translocations on the secondary population is that imposed by founder group size, which is often small (Taylor & Jamieson, 2008). These small founder groups may act to reduce genetic diversity in two ways. Firstly, the random sampling of alleles that occurs by establishing a new population from a small group of individuals from one location, may not capture all genetic variability available from that parent population. This instanteous loss of genetic diversity may be compounded by post-translocation loss of variation through inbreeding and genetic drift (Keller & Waller, 2002), especially if initial population growth is slow (Nei *et al.*, 1975).

A few studies have clarified this link by showing clear associations between translocations and a reduction in genetic diversity. Mock *et al.* (2004) used molecular techniques to elucidate the complex genetic effects of translocations of Merriam's turkey (*Meleagris gallopavo merriami*) in the south-west United States and found significant reductions in genetic diversity in translocated populations, even in populations with large founder numbers in location with high quality habitat. Tarr *et al.* (1998) used microsatellite loci to assess variation betweens translocated and parent populations of the Laysan finch (*Telespiza cantans*) and found translocated

populations had significantly lower numbers of alleles and polymorphic loci. Variations in genetic diversity between translocated populations corresponded with founder population size, with smaller founder groups resulting in greater reductions in diversity. Stockwell *et al.* (1996) examined genetic diversity in source and introduced populations of Western mosquitofish (*Gambusia affinis*) and also showed that translocations resulted in lower genetic diversity in these populations.

New Zealand's history of translocations of threatened bird species to offshore islands provides a range of genetic diversity case studies. Both neutral markers (minisatellites) and fitness-related genes (Major Histocompatibility Complex (MHC) Class II B) were used to compare genetic diversity between translocated and source populations of New Zealand robins (*Petroica australis)* as well as the severely bottlenecked population of black robin (*Petroica traversi*) (Ardern & Lambert, 1997; Arden *et al.*, 1997; Miller & Lambert, 2004a). These studies showed that, while both source and bottlenecked populations of New Zealand robins had retained some genetic diversity at both minisatellite and MHC loci (even in populations with tiny founder groups), the black robin was apparently monomorphic at the MHC Class II B locus and had the one of the lowest neutral marker diversities of any bird yet reported. These results show that comparisons between heavily bottlenecked populations and translocations using small founder groups should be used advisedly as founder groups as small as n = 5 may still provide adequate genetic diversity in the longer term (Arden *et al.*, 1997). Arden & Lambert (1997) also maintain that even species that have undergone a bottleneck as major as that of the black robin, may still persist in spite of this and that high levels of diversity are not essential for their recovery. This is also appears to be true of the Mauritius kestrel (*Falco punctatus*) (Groombridge *et al.*, 2000), which suffered a similar bottleneck.

A study of another New Zealand species, the South Island saddleback (*Philesturnus carunculatus carunculatus*), focused on sequential translocations, which impose serial bottlenecks (Taylor & Jamieson, 2008). The recovery of this bird from just 36 individuals in one remnant population to over 1,200 is remarkable and mirrors that of the recovery of the noisy scrub-bird (*Atrichornis clamosus*) (see 1.4.2). However, what is more surprising is that this is a result of sequential translocations, often using small founder groups (22 – c.400). Theoretically, each serial bottleneck will cause reduction in genetic variability through the action of random sampling of alleles, drift and potentially inbreeding. Despite this, the study found genetic variation between

populations was not significantly different, which indicates that species that are genetically depauperate prior to translocation will suffer minimal further loss of genetic diversity as the remaining alleles will persist. Futhermore, there is evidence for the stitchbird (Brekke *et al.*, 2011) (yet another New Zealand endemic) that reintroduced populations of this species have retained relatively high genetic diversity, compared to that experienced by other translocated populations of New Zealand bird species. The authors underline the importance of high population growth rates in translocated populations and also emphasise the role that high levels of extra-pair matings in a breeding system have in maintaining relatively high levels of genetic diversity. That said, the authors point out that despite maintainance of higher than expected levels of diversity, some variability has been lost and remains a consideration for future translocations.

In considering the evidence that these studies provide, it appears that the negative effects of translocations on genetic diversity may be dependent on the history of the species and the available genetic variability pre-translocation. Translocations of small founder groups will inherently capture only a subset of the overall genetic variability. Therefore, populations established from donor populations with significant variability will experience a greater difference in genetic diversity than translocated populations from genetically depauperate donor populations. Furthermore, the value of establising new populations vs. the inherent loss of genetic diversity represents a trade-off, which should be carefully considered. Still, from the evidence presented here the risks involved with translocations of small numbers of individuals is greatly outweighed by the reduction in extinction risk brought about by the establishment of a new population.

However, to maximise the chances of success of a translocation, it seems reasonable that higher numbers of founder individuals will be preferable. Several studies have modelled what they believe to be an 'ideal' number of individuals for translocation, with Briskie & Mackintosh (2004) recommending >150 individuals, while work on the yellowhead or mohua (*Mohoua ochrocephala*) by Tracy *et al.* (2011) suggests that, in a fast-growing population, ~60 individuals may be ideal, since this will capture close to 95% of available genetic variability and more than this will increase this figure by disproportionately small increments. However, Tracy *et al.* (2011) also state that the buffering effect (from mortality etc) that more individuals will provide is potentially important.

Crucially, however, the scale of translocation programs is highly resource dependent and, consequently, the number of founders in New Zealand, for example, is usually 30-40 (Briskie & Mackintosh, 2004; Tracy *et al.*, 2011), substantially less than that recommended by either of these studies. This may be compounded in species that are rare, elusive or cryptic as the resource input for the capture and movement and subsequent monitoring of single individuals will be higher than for species that are easier to capture and monitor (e.g. Sumatran rhinoceros (*Dicerorhinus sumatrensis*) vs. other rhinoceros species, (Emslie *et al.*, 2009)). In these cases (and others besides) the downstream monitoring of genetic diversity is an alternative and this can be used to inform population and genetic management strategies (Sarrazin & Barbault, 1996; Cardoso *et al.*, 2009; De Barba *et al.*, 2010). This study takes the form of just such a downstream monitoring approach and we hope to use the information obtained from this work to inform the future management of the noisy scrub-bird, including future directions for the translocation program.

1.4 Study species

1.4.1 Noisy scrub-bird

The noisy scrub-bird (NSB) (*Atrichornis clamosus* (Gould 1844)) is a small passerine, endemic to the region of south-western Australia. It is a member of the family Atrichornidae, the only other member of which, the rufous scrub-bird (*Atrichornis rufescens* (Ramsay 1867)), is confined to south-east Queensland and north-east New South Wales in eastern Australia. Morphological and phylogenetic studies have placed these taxa as a sister group to the lyrebirds (Menuridae) (Chesser & ten Have, 2007) and together they form the basal group for the Oscine-passerine radiation in Australasia (Barker *et al.*, 2004). They are a relatively primitive species, sharing characteristics with non-passerines and reptiles rather than with closer passerine relatives (Chesser & ten Have, 2007).

The NSB is listed as Endangered by IUCN (IUCN, 2012) and as Endangered (B1 & B2) by The Action Plan for Australian Birds 2010 (Garnett *et al.*, 2011) and key threats are listed as inappropriate fire regimes, predation by feral cats, introduced herbivores, weed invasion and climate change (DPaW, 2011)

NSBs are chestnut-brown in colouration and the adult male is distinguished from the female by a striking black throat and mask, contrasting with a white moustachial stripe (Pizzey & Knight, 2007). Males are generally heavier than females, with males

weighing between 47.0g and 57.0g and females between 31.5g and 39.2g (Danks *et al.*, 1996). They have reduced wing structure which limits their flying abilities considerably, but they have powerful legs which make them agile amongst vegetation and on the ground (Smith, 1985; Danks *et al.*, 1996).

Figure 1.2 Male noisy scrub-bird showing diagnostic black throat, white moustachial stripe and short wings (Alan Danks/DPaW)

The NSB earns its name from the powerful territorial song of the male (Pizzey & Knight, 2007), which easily distinguishes it from other bird species in its range (Danks *et al.*, 1996). They are renowned for their elusive nature which, combined with cryptic plumage and a preference for dense vegetation, consequently means they are rarely observed (Danks *et al.*, 1996). Nevetheless, they are an inquisitive species and will approach at close quarters without being heard or seen (Smith, 1985).

NSBs have a preference for wetter and well-vegetated areas of long-unburnt scrub and low forest habitats, particularly in gullies, drainage lines, swamps and the margins of other wetlands (Danks *et al.*, 1996). Smith (1985) characterises the ecological preference of the NSB as being the wet zone of the distribution of marri (*Corymbia callophylla*) and jarrah (*Eucalyptus marginata*) forest, especially the ecotone between forest and swamp, which holds true for much of their current and historical distribution (Figure 1.3). On Bald Island the vegetation structure is slightly different, with Bald Island marlock (*Eucalyptus conferruminata*), peppermint

(*Agonis flexuosa*) and *Melaleuca* spp. being the dominant overstorey species (Figure 1.4) and NSBs occupy more areas of understorey habitat that would be considered 'marginal' on the mainland (S. Comer & A. Danks *pers. comm.*). NSB nesting habitat requires the presence of long-leaved sedges (e.g. *Lepidosperma spp*. and *Anarthria scabra* (Smith & Robinson, 1976)) both for nesting material and nest sites, although they will also nest in other dense vegetation (Danks *et al.*, 1996). The female lays one egg which she incubates for between 28 and 46 days (Smith & Robinson, 1976), usually from late May through to early October with a peak in June (Smith 1985). If the nest fails due to the loss of the egg, the female will re-nest some distance (20-50m) away but will not re-nest if the chick is lost (Smith, 1985) or fledged (Smith & Robinson, 1976). Therefore, we can infer that in natural conditions, NSBs will be a slow-breeding species with a maximum of one chick produced per nest per year. We would also expect such low reproductive output to be associated with a relatively long-lived species.

Figure 1.3 Noisy scrub-bird habitat on Mt Gardner (including areas of *Lepidosperma* sp. sedge nesting habitat in centre right of photograph (circled) (Saul Cowen/DPaW)

Figure 1.4 Noisy scrub-bird habitat on Bald Island showing *Eucalyptus conferruminata* and *Melaleuca* sp. overstorey) (Saul Cowen/DPaW)

Little is known of the mating system of the NSB. A doctoral study by A. Berryman (unpublished; *pers. comm.)* suggested that the singing behaviour of territorial males was indicative of a polygynous system, where singing males cluster forming a discrete 'song-group', where members of this group perform the same song pattern. This situation fits the 'hot-shot' theory for a polygynous mating system, whereby males form a 'lek' around a dominant male, who controls the song group and wins the majority of matings to attendant females. Smith (1985) stated there was no direct evidence for polygamy in NSBs but males have been known to visit breeding females outside their territories and the author suggests males may be opportunistic in their polygyny. This statement was supported by evidence from a captive male NSB who mated with three different females in successive years.

Due to the cryptic nature of the NSB, this theory is conjectural at present but there is some evidence to back this idea. Polygynous mating systems place increased selection pressure on secondary sexual characteristics which increase the mating success of males including increased size and aggressive territorial behaviour (Selander, 1965), both of which are conspicuous in the NSB. Male NSBs are, on

average, nearly 50% heavier than females with a mean mass of 51.8g compared to females at 34.6g (Danks *et al.*, 1996). Size dimorphism in polygynous species has been shown in mammals (Weckerly, 1998) and is believed to be driven by sexual selection (Darwin, 1872), either through female choice or by male-male competition (Dunn *et al.*, 2001) and, theoretically, variations in size and plumage between the sexes will be highest when sexual selection is most intense, i.e. in polygamous mating systems. This disparity in mass would perhaps seem to favour a polygynous mating system. Additionally, NSBs are known for their aggressive territorial displays, which are exploited in their capture for translocation and tissue sampling (see section 2.2.2.).

Secondly, it is apparent that male NSBs play little or no part in the raising of their offspring (Danks *et al.*, 1996), another trait that can be considered typical of a polygynous or 'lek' mating system (Silver *et al.*, 1985).

Therefore, despite the lack of empirical evidence, it seems a reasonable assumption that the song sharing among groups of male NSBs is an indication of a polygynous mating system, rather than a socially monogamous system. This is a crucial factor for the estimation of effective population size (N_e) which in turn is a vital component of population genetics theory. If NSBs are a polygynous species, with a few males monopolising many females, this would reduce the N_e (Nunney, 1993) and hence the genetic diversity of the population as a whole. Through examining the genetic diversity of NSB populations, particularly on Bald Island, this study hopes to add to the knowledge of their breeding system which may influence the future management of the species.

The NSB was known to indigenous Nyoongar peoples long before the arrival of Europeans as 'Jeemuluk' (Danks *et al.*, 1996). In 1842, John Gilbert became the first European to encounter the species at Drakebrook, near what is now Waroona south of Perth and populations were later recorded from the south-west between Margaret River and Augusta and also on the south coast of Western Australia around Albany and Mt Barker (Figure 1.4), the latter region holding the greatest abundance. However, by the early 1900s the species was apparently extinct, with several extensive searches failing to find any individuals in locations where the species had been previously recorded (Danks *et al.*, 1996). The apparent extinction is believed to be have been driven by increased fire frequency and loss of habitat after European

settlement (Danks *et al.*, 1996). In 1949 a memorial, commemorating both Gilbert and the NSB, was erected at the original site of discovery.

Therefore, when a population of NSBs was discovered in 1961 on Mt Gardner near Two Peoples Bay, 30km east of Albany (Figures 1.5 & 1.6), it was particularly providential. The discovery of this previously 'extinct' species led to the gazetting of Two Peoples Bay Nature Reserve in 1967, which was then under threat of development (Orr *et al.*, 1995). The original population was estimated at 40-45 territorial males but careful management of the population helped the population to reach 179 territories in 1994 (Danks *et al.*, 1996). From 1983 until the present, translocations of birds from Two Peoples Bay have been used to try and establish new populations in a number of locations around the south-west of Western Australia (Figure 1.5). Some translocations have proved successful and new populations have been seeded around Mt Manypeaks Nature Reserve and Waychinicup National Park

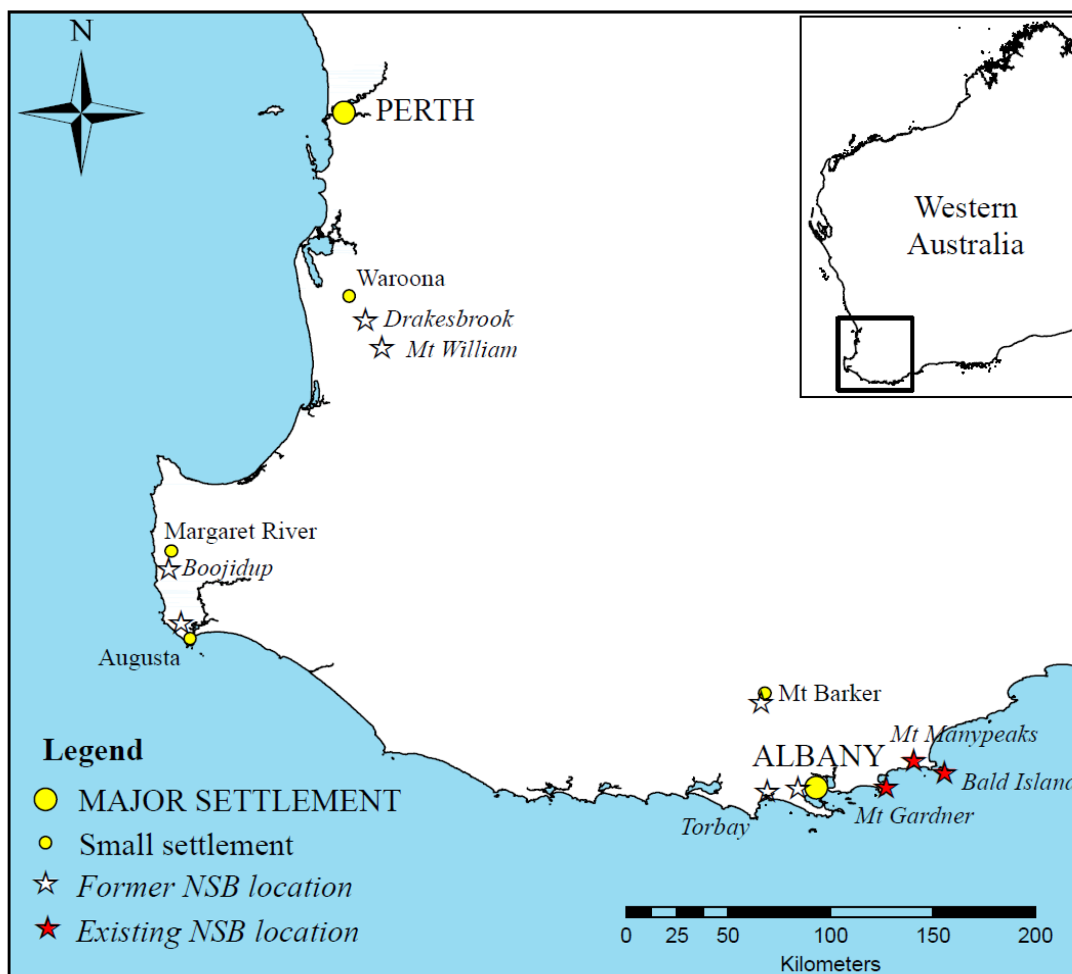


Figure 1.5 Current and former distribution of the noisy scrub-bird (adapted from Danks *et al.*, 1996)

as well as on Bald Island Nature Reserve, leading to the distribution we see today in Figure 1.6. Management of NSBs by the Western Australian Department of Parks and Wildlife (DPaW; formerly Department of Environment and Conservation) takes place on lands under DPaW tenure in the Albany area, known as the Albany Management Zone (AMZ). The AMZ is divided into discrete zones, which are largely used for population work such as censuses (Figure 1.6). However, the division of these zones is largely arbitrary and in several cases, movement of NSBs is known to occur. For example, although the Angove-Normans, Manypeaks, Waychinicup and Mermaid zones are considered discrete populations, individual NSBs frequently move between zones, for example, the post-fire exodus from Mt

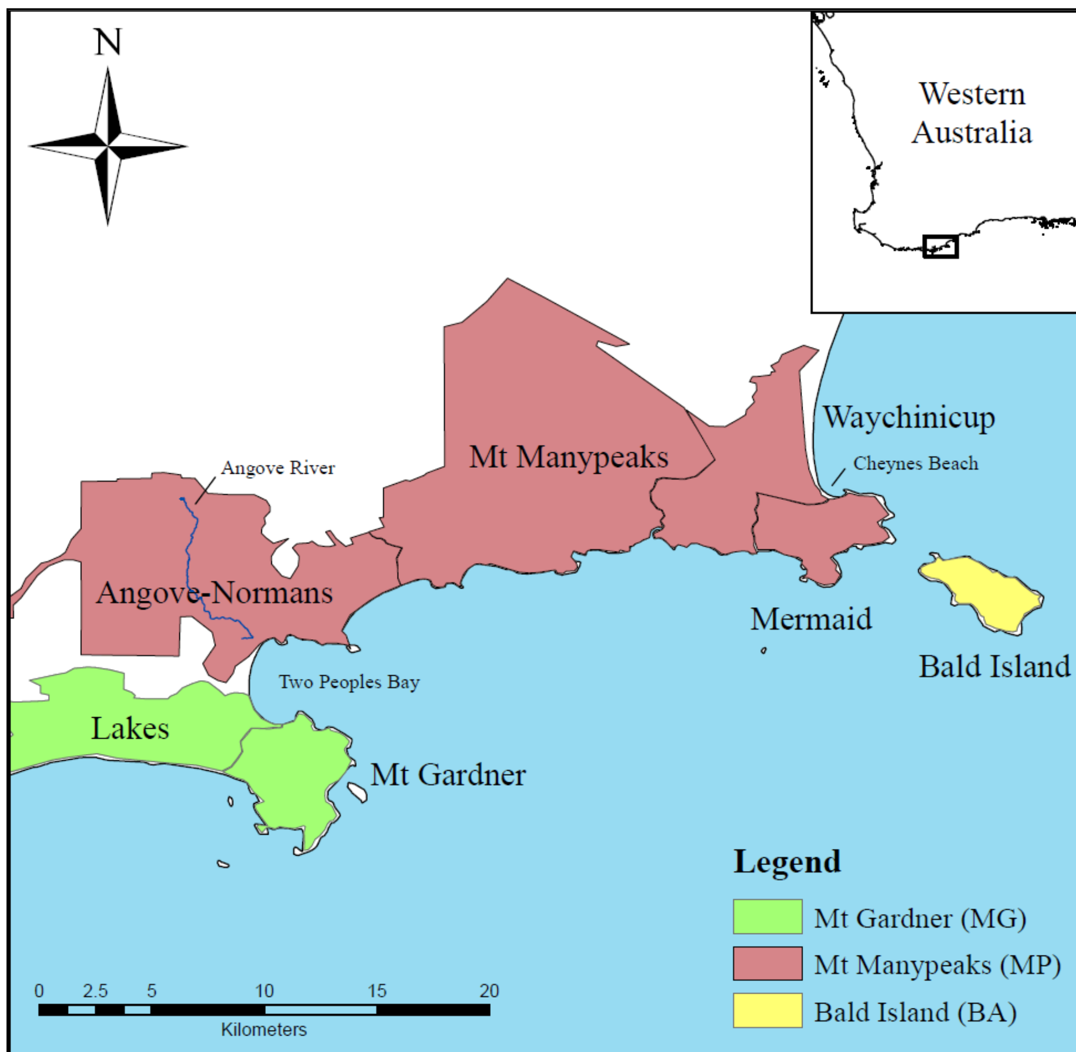


Figure 1.6 Map of Albany Management Zone (AMZ) census zones, indicating which zones are included into putative demes for the purposes of this study (DPaW)

Manypeaks in 2004/2005 where territory numbers spiked in Waychinicup immediately after the fire but then declined as refugees returned to Manypeaks as post-fire conditions improved (Figure 1.7). Therefore, for the purpose of this study, NSBs from Angove-Normans to Mermaid will be considered one genetic population or deme with no barriers to gene flow between any of the arbitrary 'zones'.However, while these four zones can be considered to be one single population, the two other zones are more safely described as discrete populations. The original population on Mt Gardner at Two Peoples Bay Nature Reserve is largely confined to the peninsula from Nanarup Beach in the west to Two Peoples' Bay in the north-east, with a thin isthmus between the two the only connection to the rest of the mainland. NSBs have dispersed through the isthmus into both the 'Lakes' area (between Moates and Gardner Lakes) to the north-west (from 1979 onwards) and to the Angove River in the north (in 1982) (Danks *et al.*, 1996), the latter being part of the Angove-Normans census zone. The occurrence of birds in this area was prior to any translocations to Mt Manypeaks so they were indisputably immigrants from Mt Gardner. In this case, these two populations could be considered meta-populations, with Mt Gardner acting as a source and the Lakes as a sink. However, numbers in these areas have declined in recent years (Figure 1.7) and since a wildfire in 2000 wiped out all territories in the Lakes area, it seems unlikely that further immigration has taken place since then. For that reason, Mt Gardner and the Lakes have been considered a separate deme to the rest of the extant mainland populations.

The third and final population considered by this study was the translocated population on Bald Island Nature Reserve. Bald Island is approximately two kilometres off the mainland of Western Australia and this presents an unfeasible barrier to NSBs since they are such poor fliers, incapable of sustained flight. Therefore, the population on Bald Island is an entirely closed system, with all NSBs on the island being descended from the original members of the translocated founder population. For this reason, Bald Island presents a fascinating example of a population derived from a relatively tiny founder group (maximum 11 individuals) that is completely free of immigration and emigration, but has still managed to thrive in a new environment (Figure 1.7). Seeking to better understand this remarkable situation will be a key element of this study. BA will be considered as the third deme.

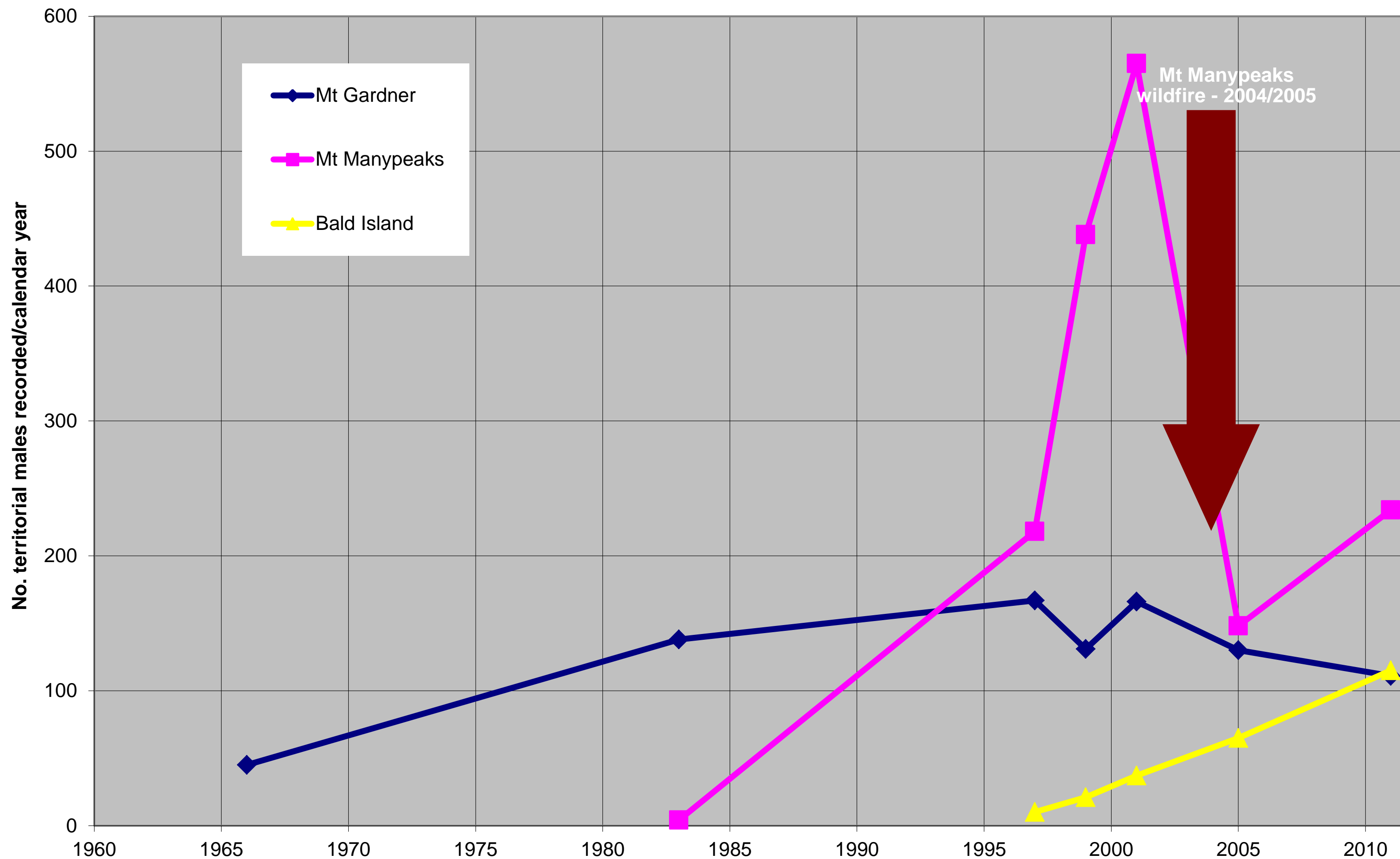


Figure 1.7 Population history of three genetic populations (demes) of noisy scrub-birds (*Atrichornis clamosus*) since monitoring began in 1966 (DPaW)

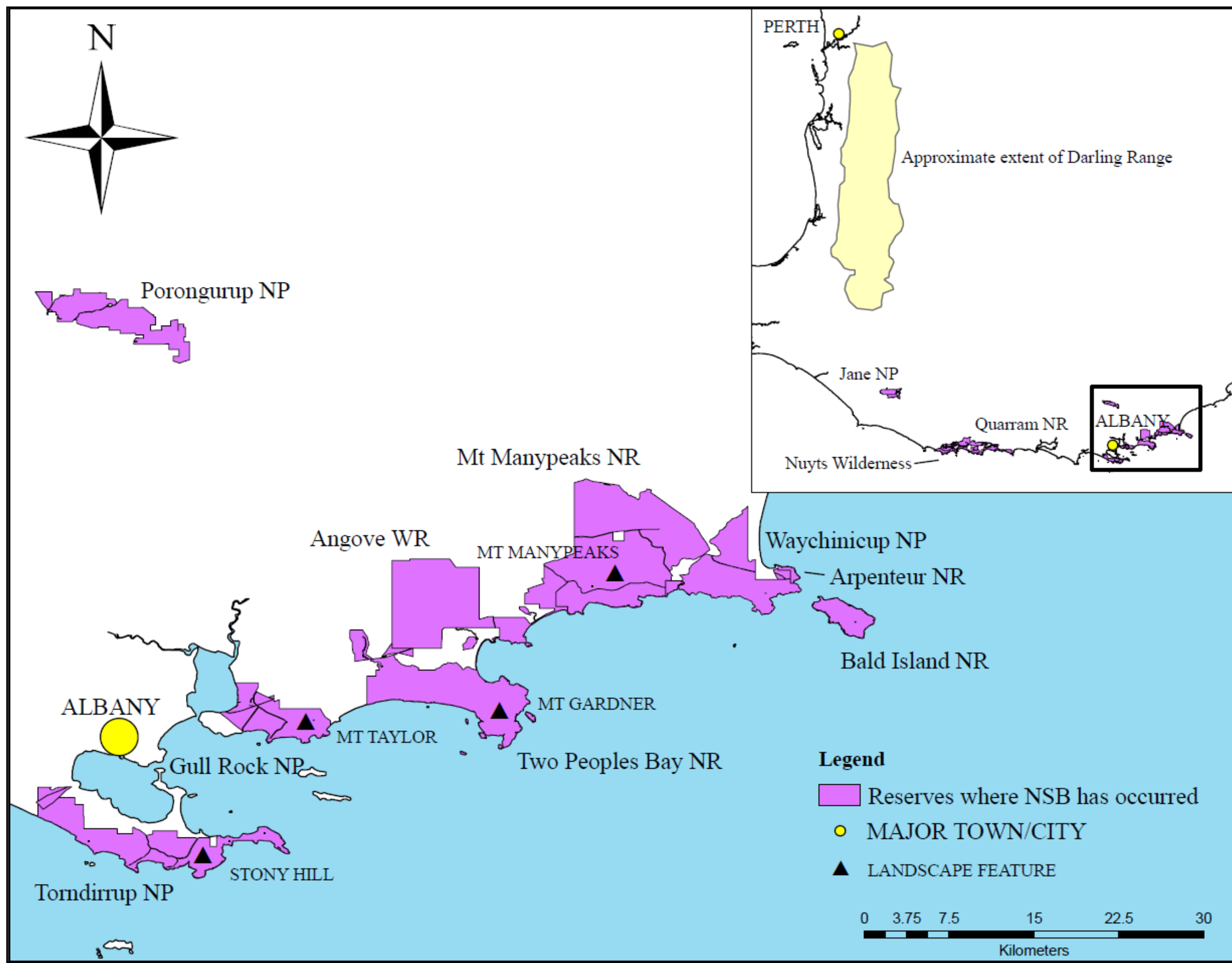


Figure 1.8 Map of locations where noisy scrub-birds have occurred naturally or through translocations (DPaW)

There are other zones within the AMZ that haven't been considered by this study. Mt Taylor is the name of the zone within Gull Rock National Park (Figure 1.8). A successful translocation in 1990 led to the establishment of a small population of NSBs at Mt Taylor. However, subsequent wildfires have extinguished this population and a further translocation to Mt Taylor in 2007 has proved unsuccessful. Therefore, this area was not considered for this study.

The final area with the AMZ which has held NSBs in recent years is Porongurup National Park (Figure 1.8), where eight male birds were released in 2006 (Tiller *et al.*, 2007). Despite a promising start, these birds were all but wiped out in February 2007 with a wildfire that burned most of the park. In 2008 only two singing males were present in the vicinity of the park and in 2009 no birds were heard. However, even if this, or the Mt Taylor translocation in 2007, had been successful, most of the birds occurring in these areas would very likely be the original translocated individuals (not their offspring) given the timescales involved.

In summary, this study considered three core areas where NSBs could be considered to be in discrete genetic populations or demes: Mt Gardner (MG), Mt Manypeaks and surrounds (MP) and Bald Island (BA) (Figure 1.6). For simplicity, NSBs occurring between the Angove River and Cheynes Beach will be referred to being part of the 'Manypeaks' or MP deme, since Mt Manypeaks is the most dominant feature of the area, both geographically, topographically and also in terms of the numbers of NSB territories recorded on and around it in the past.

Only a little was known of NSB genetics prior to this study, all from a study by J. McLoughlin (2003). This study sought to characterise microsatellite loci and assess the diversity in source and translocated populations, although sufficient samples of DNA for the analysis of individuals translocated populations were unable to be extracted. This was mainly due to difficulties in extracting sufficient quantities from feather samples and led to the recommendation that blood tissue samples were preferable for DNA analysis.

Three polymorphic dinucleotide microsatellite markers were characterised in this study, representing the first genetic markers of any kind in the species. The author suggested that for the original population at Mt Gardner, despite some loss of genetic diversity, significant diversity may still exist. However, given that this study only used three dinucleotide loci for one population, it is difficult to form robust conclusions based on these data. In an effort to build on this early work, this study

will concentrate on characterising at least 10 tetranucleotide microsatellites which will provide more meaningful data and finer-scale resolution.

An important aspect of NSB population genetics are the sizes of the founder groups used in the translocations. Founder size is crucial for the genetic diversity of the translocated population and, as shown by Table 1.3, NSB founder sizes have tended to be small, even in those populations that have been successful in the longer term (e.g. Manypeaks and Bald Island) and fall below the thresholds suggested in 1.3.2. Furthermore, the presumed 'extinction' of the NSB c.1900 and its subsequent rediscovery in 1961 at Two Peoples Bay, may represent a significant population bottleneck. Therefore, the small founder groups taken from this population to seed new ones may exacerbate the potential problems associated with long-term bottlenecks (e.g. Mock *et al.*, 2004). This will be a key facet of this study of the conservation genetics of the NSB.

Table 1.3 Translocation founder group sizes of noisy scrub-birds ((from Comer *et al.*, 2010)

Location	Translocation Years	Males	Females	Total
Mt Manypeaks*	1983-1985	18	13	31
Nuyts Wilderness†	1986-1987	16	15	31
Quarram NR	1989-1990	15	11	26
Mt Taylor‡	1990-1992	6	6	12
Bald Island*	1992-1993	8	3	11
Mermaid*§	1992-1994	8	2	10
Stony Hill‖	1994	5	0	5
Darling Range	1997-2003	60	20	80
Porongurup NP	2006	8	0	8
Mt Taylor‡	2007	5	0	5
Jane NP	2010	5	1	6

* populations that are currently extant; † part of Walpole-Nornalup NP; ‡ part of Gull Rock NP; § Arpenteur NR and part of Waychinicup NP; ‖ part of Torndirrup NP (see Figure 1.8)

1.4.2 Barrow Island white-winged fairy-wren

The white-winged fairy-wren (*Malurus leucopterus,* (Dumont 1824)) is a small passerine and a widespread member of the family Maluridae (Pruett-Jones & Tarvin, 2001), which includes the fairy-wrens, grass-wrens and emu-wrens.

Figure 1.9 Male Barrow Island white-winged fairy-wren showing black (rather than blue) and white breeding plumage (Alan Danks/DPaW)

While the male of the mainland subspecies *M. leucopterus leuconotus* is vivid blue-and-white in full breeding plumage, the island subspecies of *M. l. leucopterus* (Dirk Hartog Island, Western Australia; Figure 1.10) and *M. l. edouardi* (Barrow Island and the Montebello Islands, Western Australia; Figure 1.10) are black-and-white (Figure 1.9) (Driskell *et al.*, 2010) and are also known as the black-and-white fairy-wren (BWW). Both *M. l. leucopterus* and *M. l. edouardi* are both listed as Vulnerable (D2) by The Action Plan for Australian Birds 2010 (Garnett *et al.*, 2011) since both subspecies occur in single populations at risk from large-scale fires and introduction of alien predators.

On Barrow Island, BWWs are the second most abundant bird species with c.8000 individuals (Pruett-Jones & O'Donnell, 2004) and are found in most habitat types but appear to be most abundant on ridges with *Triodia wiseana* mixed with open and dense shrubby vegetation (Pruett-Jones & Tarvin, 2001). In this respect *M. l. edouardi* is ecologically quite similar to the mainland subspecies.

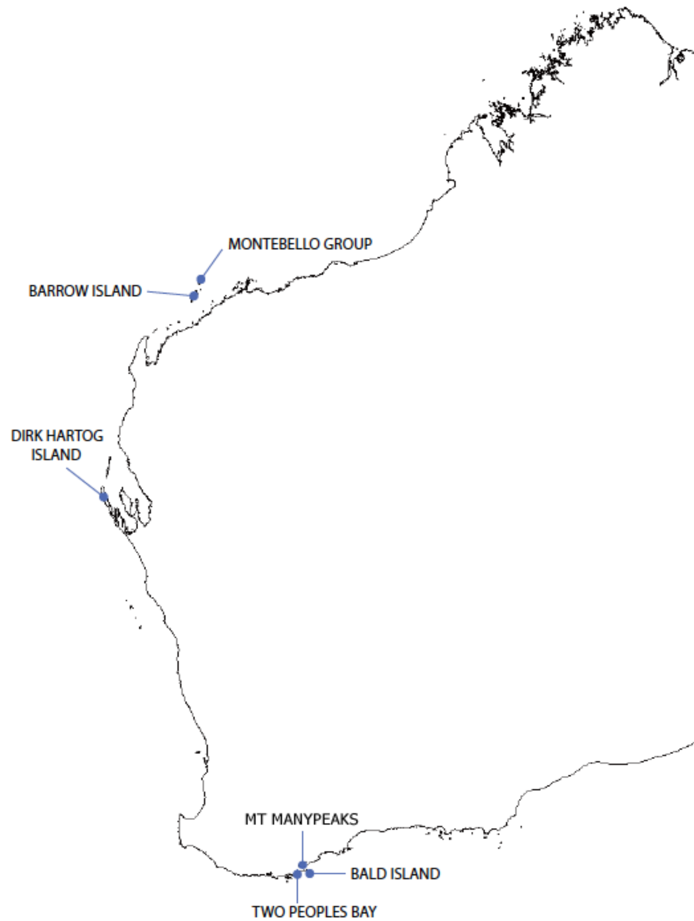


Figure 1.10 Locations of study populations of noisy scrub-bird (*Atrichornis clamosus*) (Two Peoples Bay/Bald Island), white-winged fairy-wren (*Malurus leucopterus leucopterus* (Dirk Hartog)/*Malurus leucopterus edouardi* (Barrow Island/Montebello Group)) and spinifexbird (*Eremiornis carteri*) (Barrow Island/Montebello Group) within Western Australia (adapted from University of Melbourne 1994-2011)

However, breeding ecology may differ somewhat between these taxa, since *M. l. leuconotus* is a cooperative breeder with a 'clan' mating system and studies on Dirk Hartog Island have shown *M. l. leucopterus* to be largely socially monogamous (Rathburn & Montgomerie, 2003). Studies on Barrow Island have suggested that *M.l. edouardi* may also be socially monogamous rather than cooperative, but this is based on limited evidence (Pruett-Jones & Tarvin, 2001). This may, in turn, have an impact on the effective population size (N_e) of this population, in comparison to the mainland subspecies. Rathburn & Montgomerie (2003) cite the work of Griffith (2000) who found that extra-pair copulations were significantly less common in island than mainland populations, due to decreased sexual selection on islands. This fact may explain why cooperative breeding does not occur on Dirk Hartog and, by

extrapolation, Barrow Island, since there is little advantage in related individuals acting as helpers if there are fewer opportunities for extra-pair copulations. Fewer extra-pair copulations will also have an impact on N_e and hence genetic diversity as N_e is strongly linked to mating system type (Nunney, 1993).

Historically, *M. l. edouardi* was not solely confined to Barrow Island but also occurred on Trimouille Island in the Montebello Group, NNE of Barrow (Burbidge *et al.*, 2000). However, the species became extinct after the introduction of the black rat (*Rattus rattus*) and feral cat (*Felis catus*) to the islands and efforts were made to eradicate these species to facilitate reintroductions of *M. l. edouardi* and spinifexbird (*Eremiornis carteri*) (Algar *et al.*, 2002; Burbidge, 2004). In 2010, both species were reintroduced to Hermite Island (Garnett *et al.*, 2011) and this translocation, as well as another in 2011, provided this study with tissue samples for DNA analysis.

Phylogenetic work from Driskell *et al*. (2002) found that *M. l. edouardi* diverged from *M. l. leuconotus* and *M. l. leucopterus* in the Late Pleistocene 220,000-245,000 yrs ago, consistent with a post ice-age sea-level rise before which a land-bridge would have connected Barrow Island with mainland Australia. The authors also suggest that subsequent sea-level changes would have resulted in land-bridges, unsuitable coastal habitat which may have acted as a barrier to mixing of populations. This long-term geographic isolation is relevant from a genetic viewpoint and will be discussed later.

Another study also found that the black colouration of breeding males found in the two island subspecies evolved in isolation from blue pigmented birds (i.e. *M. l. leuconotus* on mainland Australia) on both Barrow and Dirk Hartog in separate events (Driskell *et al.*, 2010), although why this may have occurred is not clear. Finally, low genetic differentiation was found between all three subspecies but there was support for western *M. l. leuconotus* and the two island subspecies forming a group distinct from eastern *M. l. leuconotus*, although this was not supported by bootstrapping.

The long-term isolation of *M. l. edouardi* is relevant for this study, since we can predict that the lack of gene-flow will have led to the loss of genetic diversity through drift (Keller & Waller, 2002). Therefore, we might expect *M. l. edouardi* to have less genetic variation than mainland birds of the subspecies *M. l. leuconotus*, or for that matter the nominate subspecies *M. l. leucopterus* on Dirk Hartog, which has been shown to have interbred with *M. l. leuconotus* more recently (Driskell *et al.*,

2002). Additionally, the evolution of the black pigmentation in male breeding plumage from blue also raises interesting questions regarding the reasons why this has occurred. One theory is that it might be related to the differences observed in mating systems in mainland and island populations and this would potentially have an associated influence on effective population size and genetic diversity.

1.4.3 Spinifexbird

The spinifexbird (SPB) (*Eremiornis carteri* (North, 1900)) is a small passerine of the family Locustellidae (formerly part of the Old World Warblers (Sylviidae)) that is widespread across northern Australia in areas of *Triodia* spp. spinifex grasses (Wooller & Bradley, 1981). It is the most abundant bird species on Barrow Island (Figure 1.10) with approximately 25,000 individuals present on the island (Wooller & Bradley, 1981; Pruett-Jones & O'Donnell, 2004).

SPBs are thought to be both seasonal and opportunistic breeders that will breed in response to heavy rains (e.g. cyclones) (Ambrose & Murphy, 1994) and are usually seen in groups of two (Wooller & Bradley, 1981), suggesting that they are socially monogamous, although apparently little is known of their breeding behaviour. It is a weak flier (Ambrose *et al.*, 1996) and is usually a shy and cryptic species, aided by

Figure 1.11 Adult spinifexbird showing light-brown colouration, aiding the often cryptic behaviour of the species (Alan Danks/DPaW)

its light-brown colouration (Figure 1.11) and consequently rarely observed away from its apparent stronghold on Barrow Island (Wooller and Bradley, 1981).

The SPB was previously known to occur on Hermite and Trimouille Islands in the Montebello Group 20-40km NNE of Barrow Island but became extinct (Burbidge *et al.*, 2000). The eradication of black rat (*Rattus rattus*) and feral cat (*Felis catus*) from the islands has allowed reintroductions of SPB and BWW (Algar *et al.*, 2002; Burbidge, 2004). These reintroductions have provided tissue samples for DNA analysis in this study.

Chapter 2 – Materials and methods

2.1 Population census

2.1.1 Aims of surveys for census

Annual census surveys of NSBs are used to provide a population index (Smith & Forrester, 1981), which is used to monitor population changes and trends over a period of more than 50 years from when the species was rediscovered in 1961 (Danks *et al.*, 1996). These trends can be used to inform management decisions for these populations and the areas they occur in. The observed trend lines allow potential population declines to be quickly identified and acted upon, which is particularly important in the maintenance and success of translocated populations (Danks *et al.*, 1996). However, this is also relevant for populations that are used as sources for translocations, where individuals are captured and removed from the population. For example, the decline in singing male NSBs on Mt Gardner up to 1999 led to the halting of the use of this population as a source for translocations (Comer & Danks, 2000). Therefore, the census of populations that are used as sources for translocations is also a high priority.

A secondary aim of annual census surveys is to identify and locate active male territories. This provides information on the spatial and temporal activity of male NSBs in any one season and these data are comparable between annual censuses. This serves as an indicator of the general suitability of the habitat for NSBs (Smith 1985), which is hypothesised to vary spatially and temporally according to a range of factors, not least fire age (or years since burned). For example, the differential use of vegetation by individuals dispersing from Mt Gardner may suggest that this locality does not actually represent optimal habitat for the NSB (Danks, 1997).

The NSB's cryptic habits make it a very difficult species to observe readily (Smith & Forrester, 1981), with sighting-based surveys being of minimal use as an index of population size. Fortunately, the powerful territorial song of the male NSB is audible at a distance of over one kilometre (Smith & Forrester, 1981), making it a much more suitable cue for recording active territories. Therefore, the main survey technique in this species focusses entirely on recording territorial singing males (Danks *et al.*, 1996). Only males vocalise in this way and to the trained ear

vocalisations are readily distinguished from practically all other Australian bird species (Danks *et al.*, 1996).

2.1.2 Survey locations

NSB surveys have been conducted almost annually since 1966, five years after the species was rediscovered on Mt Gardner at Two Peoples Bay Nature Reserve. Since 1970, this subpopulation[2] has been surveyed almost every year (excluding 1978 and 1981) (Danks *et al.*, 1996) and represents the only 'original', and hence most significant, subpopulation (both from a management and genetic point of view) of this species in the entire region. The Mt Gardner subpopulation is broken up into 15 individual census zones to facilitate comprehensive coverage of the subpopulation (Figure 2.1).

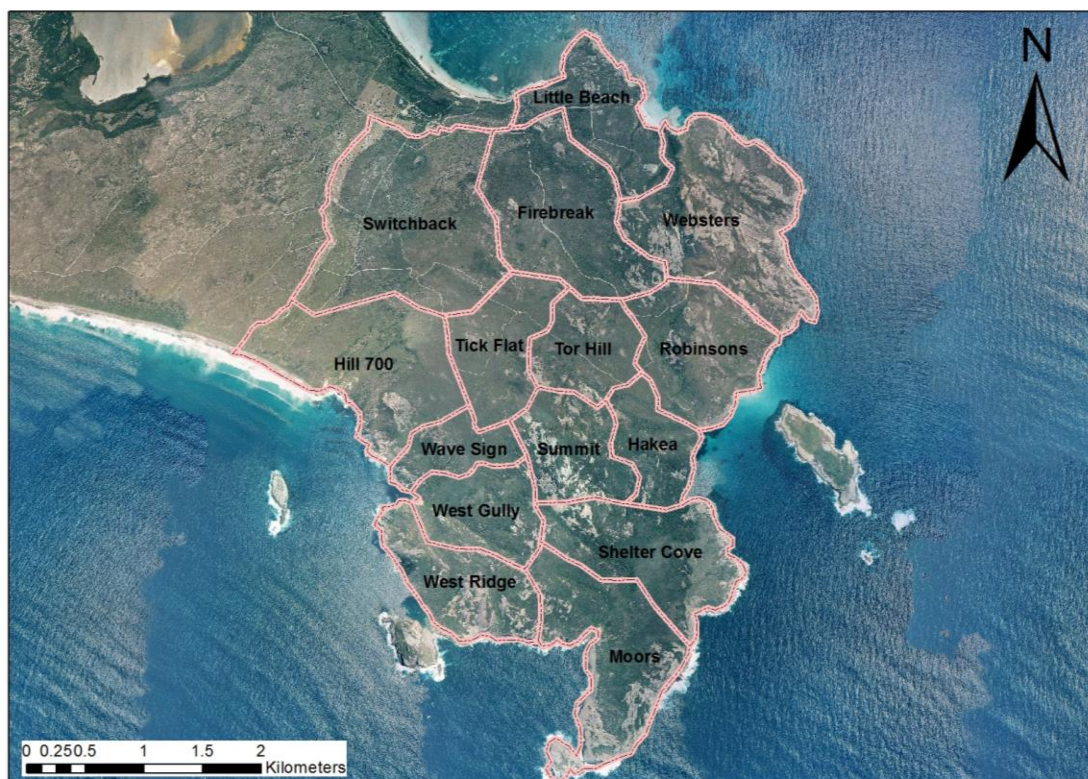


Figure 2.1 – Map of Mt Gardner area of Two Peoples Bay Nature Reserve showing noisy scrub-bird census zones (DPaW)

Surveys of other NSB populations have been carried out less frequently, often on an *ad hoc* basis, depending upon the availability of funds and personnel. These

[2] In the context of management, discrete populations will be referred to as 'subpopulations' rather than 'demes', since current management doesn't take genetic populations into consideration.

populations are broken up arbitrarily into zones so that they can be more easily covered (Figure 1.6). The area from north of Two Peoples Bay to Cheynes Beach is broken up into five separate zones. Bald Island occupies its own census zone. Additionally, the area of Two Peoples Bay to the north and west of Mt Gardner is designated as a separate census zone, known as Lakes due to the proximity to Moates and Gardner Lakes. These combined census zones comprise the management area known as the Albany Management Zone (AMZ) (see 1.4.1).

2.1.3 Survey methodology

Surveys for territorial male NSBs are carried out during the breeding season from May to September and generally take place in the early morning when calling frequency is highest. Surveys incorporate walks around reserves and other localities where NSBs occur, focussing particularly on previously recorded territories, as the same territory has been shown to be occupied consistently for many years. Additionally, particularly in new and expanding populations, any other areas of suitable habitat are included in the survey.

The powerful call of the male NSB can be heard at significant distance (>400m) and when calling is heard the direction (compass bearing) and an approximate distance are recorded. A GPS location of the position the bird was heard from is taken and plotted on a map (using a recent satellite orthophoto) and from this location the position of the bird can be estimated. Often it is necessary to take two or more bearings on a calling bird to ensure accurate positioning. Only birds giving the distinctive territorial song are recorded as the population index is of territorial birds (subordinate birds may also give a variety of other calls but rarely territorial song) and also it avoids confusion with other species.

2.2 Capture and tissue sampling

2.2.1 Aims of tissue sampling

In order to provide this study with an insight into genetic diversity in all three extant subpopulations of NSBs, samples of tissue were required from the translocated population on Bald Island, which had not been previously sampled since it was established. Optimal sample size was selected using the formula of Lowe *et al.* (2004): $P[A^+_1, A^+_2] = 1 - (1 - p_1)^n - (1 - p_2)^n + (1 - p_1 - p_2)^n$

Where A_1 and A_2 are alleles and p_1 and p_2 are the frequencies of each allele, $P[A^+_1, A^+_2]$ is the probability that a random sample of *n* gametes contains at least one of each allele. This finds that for a pair of alleles of which the most common has a frequency of 80%, to ensure both alleles are sampled in a population, a minimum of 20 samples are required. If the frequency of the allele is 95%, then ~59 samples are required. Since the capture of 59 individual scrub-birds is unlikely, the desired sample size was at least 20 animals and a maximum of 60 animals.

Tissue samples from BWWs and SPBs were obtained from individuals captured as part of a translocation from Barrow Island to Hermite Island in the Montebello Group. Samples of blood feathers were taken, ostensibly for gender determination work, but the subsequent extracted DNA samples obtained for this purpose were all used in this study.

2.2.2 Capture methodology

The capture of live NSBs for translocation has been a fundamental part of the conservation management strategy for this species since the 1980s (Burbidge *et al.*, 1986, Danks *et al.*, 1996) and individuals used in this programme have had tissue samples taken for subsequent DNA analysis. Due to the cryptic and shy nature of this species, passive capture (e.g. not using call-playback) techniques have not proved suitable. Hence, a specific technique has been developed for the capture of individual NSBs using modified mist-nets on areas of bare ground or 'netlines'. Territorial male birds are easily discerned and located by their loud territorial song and this allows a suitable site for a netline to be located. Ideally, netlines should be located in dense vegetation of at least 1.5 metres high with some canopy cover. This minimises the amount of light and wind on the net, which may allow birds to see the net more clearly, which reduces the likelihood of capture success. Favoured vegetation types for netlines in this study usually included *Thomasia* spp., *Melaleuca* spp. and *Acacia* sp., often with *Agonis flexuosa* overstorey, although this varied with the primary location (in this case Bald Island).

Netlines must be clear of any material that may snag a net as the bottom shelves of the net are laid horizontally on the ground (see Figure 2.1) and hides must be established at either end of the netline for the capture team to use during a capture attempt.

Figure 2.2 - Volunteers and Department of Environment and Conservation staff practice setting up a modified mist-net for noisy scrub-bird capture (N.B. this site was a not a capture attempt location) (Sarah Comer/DPaW)

Once a netline is established and the mist-net erected as illustrated in Figure 2.2, audio speakers are secreted on either side of the net and connected via cable to an audio device (e.g. mp3 player) which is controlled from a hide at one end of the net ('playback' end). This audio device is used to entice territorial birds into the vicinity of the netline by playing NSB calls, although only through a single speaker at a time. Territorial birds will approach the speaker, responding aggressively with their own alarm calls and territorial song and at this point the active speaker will be alternated to draw the bird across the netline. The lower shelves of the net contain a string running along each outer edge, and these run to the hide located opposite the 'playback' end ('string-pull' end). When the strings are pulled tight, the lower shelves close vertically and, when timed to meet a NSB attempting to cross the netline, will often trap the bird in the net. It is usually the responsibility of the individual at the 'playback' end to ensure the successful capture of the bird by retrieving it from the net before it can escape. Although this technique is not 100% successful, it has proven the most efficient way of capturing significant numbers of NSBs for translocations (Danks, 1994).

Previous capture attempts with the NSB have used Elliott live traps (Upwey, Victoria) to successfully capture individuals of range of ages and sexes. This

technique was used on Bald Island in 2011 combined with the use of a ‘drift-fence’ (see Figure 2.3) to encourage individuals to approach the traps. However, while two ‘drift-fences’ of approximately 20 metres length each were opened daily for seven days, no capture success was recorded.

Figure 2.3 - example of 'drift-fence' on Bald Island Nature Reserve. Live traps were inserted in holes at base of mesh when fence was 'live' (Saul Cowen/DPaW).

On Barrow Island, SPBs and BWWs were caught using traditional mist-net techniques but using call-playback to lure individuals into the net (A. H. Burbidge *pers.comm.*).

2.2.3 Tissue sampling methodology

Individual NSBs captured on Bald Island in 2009, 2010 and 2011 had tissue samples taken for subsequent DNA extraction and analysis. In 12 individuals, blood samples were taken directly from the ulnar wing vein. The vein was pierced using a hypodermic needle and blood collected using a non-heperinised capillary tube. Blood droplets were then deposited into an EDTA vacutainer and stored in a fridge or other cool, dark environment until transfer to a -20°C freezer for long term storage. In three birds, blood samples were unable to be obtained and blood feathers or large feathers containing a large quantity of pulp were collected for DNA extraction.

For SPBs and BWWs, blood feathers were taken (usually 2-5) and stored in 5ml of ethanol. This provided sufficient DNA for subsequent analysis.

2.2.4 *Summary of capture work*

On Bald Island in 2009, 2010 and 2011 a total of 15 individuals were captured for DNA sampling. Table 2.1 below shows which individuals were captured and which types of tissue samples were obtained from each bird.

Table 2.1 - Individual noisy scrub-birds captured and sampled on Bald Island

ID Code	Year	Sex	Breeding status	Tissue sampled	Fate
09M01	2009	M	Territorial	Blood Feathers	Released at home
09M02	2009	M	Territorial	Blood	Released at home
09M03	2009	M	Territorial	Feathers	Escaped at home
10M01	2010	M	Territorial	Blood	Translocated (Jane NP)
10M02	2010	M	Territorial	Blood	Translocated (Jane NP)
10M03	2010	M	Territorial	Blood	Translocated (Jane NP)
10M04	2010	M	Territorial	Blood	Translocated (Jane NP)
10M05	2010	M	Territorial	Blood	Translocated (Jane NP)
10F01	2010	F	Territorial	Blood	Translocated (Jane NP)
11M01	2011	M	Territorial	Blood	Translocated (Angove)
11M02	2011	M	Territorial	Blood	Translocated (Angove)
11M03	2011	M	Territorial	Blood	Translocated (Angove)
11M04	2011	M	Territorial	Blood Feathers	Translocated (Angove)
11M05	2011	M	Subordinate	Blood	Translocated (Angove)
11M06	2011	M	Territorial	Blood	Translocated (Angove)

Samples from SPBs (n = 49) and BWWs (n = 35) (see Appendix ii) were provided from individuals captured on Barrow Island in 2010 and 2011 as part of a translocation to Hermite Island. However, confusion over labelling of tubes containing BWW tissue (which was elucidated by conflicting sex determination results (D. Groth *pers. comm.*)) meant data from three individuals were excluded from the subsequent analysis

2.3 DNA extraction

2.3.1 *DNA extraction methodology (blood)*

NSB DNA was extracted from blood samples using both Qiagen DNeasy Blood and Tissue and AXYGEN AxyPrep Blood DNA Miniprep kits, depending on the

availability of either, although the Qiagen appeared to produce the greater yield and was preferable. The manufacturers' protocol was used (Appendix iv) but occasionally it needed to be modified due to the nature of the samples used. Blood samples were kept in two different forms, either in a non-heparinised capillary tube or in EDTA. Samples in EDTA were dissolved in 200µl Phosphate Buffer Solution (PBS) prior to starting the extraction protocol which was as per the manufacturer's instructions. Capillary tube samples were also dissolved in PBS in a 1.5ml microcentrifuge tube (AXYGEN) but the nature of the clotted blood in the tube required breaking it into several smaller pieces. Often the nature of clotted blood required that the sample be divided into up to four separate tubes so as to facilitate a more rapid digestion by Proteinase K. Since there was an unusually large quantity of clotted material in these tubes, it was often necessary to incubate at 56°C for a longer time period than stated in the protocol. This was occasionally overnight.
DNA was quantified by NanoDrop (Thermo Fisher Scientific) and in instances where a more concentrated sample was required this was achieved through partial drying by incubating in a 60°C oven with the tube-lid open.

2.3.2 DNA extraction methodology (feathers)

The extraction of DNA from feather tissue required a different protocol, using XytXtract – Animal (http://www.xytogen.com.au) (Castalanelli *et al.*, 2010). A small fragment (>3mm) was snipped off the base of the shaft (calamus) of the feather with a clean pair of scissors (usually in several pieces to maximise the surface area available) into a 1.7ml microcentrifuge tube. Two hundred and twenty µl of XytXtract solution 1 was added to the microcentrifuge tube with 780µl of ultra-pure water to make Buffer S1. 20µl of Buffer S1 was added to the feather sample, which was then incubated at 99ºC for 15mins. Buffer S2 (6µl) was added and then the sample was centrifuged briefly to bring down the condensation from the top of the tube. The sample was then suitable for use directly in a PCR or stored by freezing at -20ºC. However, it was found that storage of XytXtract-extracted samples in a frost-free or self-defrosting freezer greatly increased the rate at which DNA degradation occurred, due to the periodic defrosting phase of the freezer. It is recommended to store all extracted DNA samples, but especially XytXtract-extracted samples, in a non frost-free freezer.

2.3.3 *Gel extraction methodology*

In some cases, high numbers of cycles were necessary for sufficient DNA amplification by Polymerase Chain Reaction (PCR) (see 2.4) to be used in subsequent ligation and cloning reactions. However, these extra cycles caused a significant appearance of a primer dimer artefact, and it was observed that during ligation these fragments preferentially ligated into plasmids with respect to the target fragments (600-1000bp). This is most likely due to their relative size and abundance (<50bp). To overcome this problem, the target fragments were extracted from agarose after being run on an agarose gel (in TAE buffer). This methodology was performed only when using products amplified using Major Histocompatibility Complex loci primers, as standard cycle numbers were sufficient for microsatellite amplification.

To achieve the purification, the entire 20µl PCR reaction mix was incubated with 3µl of 5x Loading Buffer for 5 minutes at 60ºC, loaded into a well on an agarose gel (2%) and run at 60V for up to 90mins to attain sufficient separation of fragments. The gel was pre-stained with CybrGreen (Invitrogen). Bands for extraction were initially viewed using an ultra-violet (UV) transilluminator (door closed) to confirm successful amplification of the target fragments. For extraction of bands, it was necessary to visualise the gel under UV with the transilluminator door open so appropriate personal protection equipment was worn, including a UV face-shield and helmet. To avoid loss of T-tails of amplified fragments (which are necessary for successful ligation into T-easy plasmid vectors (see 2.7.1)) lengthy exposure of the gel to UV was avoided. Bands from individual wells were removed from the gel using a scalpel and transferred to a 1.5ml microcentrifuge tube. Extraction of the PCR product used a QIAquick Gel Extraction Kit (Qiagen) (see Appendix v) and used the standard protocol provided in the kit. Five µl of the extracted eluate was run on an agarose gel (1%) to confirm successful extraction of the desired fragments, prior to use in a ligation reaction.

2.4 Polymerase chain reaction

2.4.1 *Polymerase chain reaction methodology*

DNA amplification by Polymerase Chain Reaction (PCR) on a thermocycler was an essential tool in this study and several protocols with differing parameters were used

for various components. These protocols can be found in Appendix vi. Two different types of Taq DNA Polymerase were used over the course of this study, FastStart (Roche) and MyTaq (Bioline), and consequently two different master mixes were prepared for using each of these brands. FastStart Taq DNA Polymerase is supplied with dNTPs, $MgCl_2$ and 10X Taq Buffer which were used in the master mix described below (in 200µl aliquots) for use with it. Bovine Serum Albumin (BSA) was added to the master mix to minimise inhibition of amplification.

Table 2.2 Components of master mix for DNA amplification by PCR

Component	Concentration	Volume/200µl (µl)
dNTPs	100mM	4
$MgCl_2$	50mM	6
10X Taq Buffer	n/a	20
BSA	20mg/ml	2
Ultra-pure H_20	n/a	168

MyTaq is supplied with 5X Reaction Buffer which contains optimal concentrations of dNTPs and $MgCl_2$. Two hundred µl aliquots of master mix were made by adding 40µl of 5X Reaction Buffer to 160µl of ultra-pure water and mixing. Whereas 2µl of template DNA (20ng/µl) per 20µl reaction mixture was usually optimal for amplification with FastStart, only 1µl per 20µl was required for the same performance with MyTaq.

Most PCRs were carried out on an Eppendorf Mastercycler using protocols shown in Appendix vi. The products of PCRs were viewed by agarose gel electrophoresis.

2.5 Primer design

2.5.1 Microsatellite primer design methodology

Microsatellite loci were identified by reads from a 454 Genome Sequencer FLX (Roche Life Sciences) in FASTA format. Reads containing tetranucleotide tandem repeats of suitable length (>5 repeats) with suitably-sized flanking regions (>50bp) were imported into PRIMER3 version 0.4.0 (Rozen & Skaletsky, 2000). Primer pairs were designed using the following criteria: optimal melting temperature (T_m) of 60.0°C; 20-80% GC content & optimal primer size of 20bp. The target region was specified as the region of tandem repeats and the number of primers to return was set

at five. Each primer pair was then screened for self-complementarity, self-dimerization and hair-pin loops using OLIGOCALC (Kibbe, 2007). Primer pairs that passed all three of these criteria were then ordered for testing.

2.6 Capillary electrophoresis

2.6.1 Capillary electrophoresis methodology

Fragment analysis of microsatellite loci by capillary electrophoresis was performed using a Beckman Coulter CEQ 8000. Fluorescent-labelled forward primers were combined with non-labelled reverse primers and amplified by PCR prior to analysis. PCR parameters were optimised with non-labelled forward and reverse primer pairs and this was used to identify polymorphisms for the loci under analysis. Amplification of microsatellite loci with fluorescent-labelled primers was confirmed by gel electrophoresis (agarose).

A master mix was prepared for use on the CEQ 8000 using CEQ Sample Loading Solution (SLS) and CEQ Size Standard (400) (Beckman Coulter). The following reaction mixture (per 40µl reaction) was determined empirically and the optimal conditions are described in Table 2.3.

Table 2.3 Constiuent parts of reaction mixture for capillary electrophoresis on CEQ 8000

Component	volume/well (µl)
Sample Loading Solution	38.75
Size Standard (400)	0.25
PCR Product	1

Mixing of master mix was done by careful pipetting and all reagents were kept on ice to avoid degradation. PCR product was initially added into each well on a 96-well PCR microplate (AXYGEN) and master mix added and overlaid with a droplet of mineral oil to prevent evaporation. A corresponding 96-well immunoassay plate (Beckman Coulter) was loaded with CEQ Sequencing Separation Buffer (Beckman Coulter) in an identical pattern as the PCR product containing plate. Only complete rows on the plate were run.

Prior to loading of the microplate and immunoassay plate into the CEQ 8000, sample data was entered into the CEQ 8000 software (Beckman Coulter). Plates were loaded and the wetting tray refilled with ultra-pure water (NB this step must be completed

within 15 minutes to prevent air bubbles forming in capillary). Finally, the gel cartridge was either loaded or checked to confirm that sufficient gel was available for the run and replaced if not. After a final check, the fragment analysis was run. Spectrograms of post-run results were examined using the CEQ 8000 software package (Beckman Coulter) and were manually screened for errors. Fragment sizes determined by the software were manually ented into to an Excel (Microsoft) spreadsheet.

2.7 Transformation of competent cells

2.7.1 Ligation of PCR product into plasmid vector

A T-easy cloning kit (Promega) was used for the preparation of vectors using the standard protocol (see Appendix vii). The T-easy kit ligates an A-tailed PCR product into a ~3015bp plasmid and is selected using Ampicillin combined with blue-and-white cloning screening and requires the addition of an adenosine (A) base to the 3' terminal of an amplified sequence to complement the thymidine (T) overhang on the insert site on the vector. Vector–insert ligations were generally performed overnight at 4ºC to maximise the efficiency of ligation, as per the standard protocol.

2.7.2 Transformation of competent cells

ElectroSHOX (Bioline) electro-competent cells were transformed. To minimise 'popping' of the cuvette due to high salt content in the reaction mixture, ligation mixtures were purified using ethanol precipitation. A 2.5 times volume of 100% ethanol was added to the ligation mixture and the solution was incubated at -20ºC overnight using a 0.65ml microcentrifuge tube. The tube was then centrifuged at 14,000rpm for 30mins. The supernatant was pipetted out and discarded whilst holding the tube in the same orientation and ensuring only the top side of the tube was touched by the pipette tip. One hundred µl of 70% ethanol was added and the tube centrifuged for 10mins at 14,000rpm. The supernatant was then pipetted and discarded as before. Any remaining ethanol was removed by inverting tubes on a paper towel on a bench for 5mins. Finally, 5µl of ultra-pure water was added to resuspend the plasmids and the ligation mixture was then ready for use (or was frozen at -20ºC). Transformations were carried out as per the standard protocol (see Appendix viii) in 1mm electroporation cuvettes using a Biorad micro-pulser.

After incubation at 37ºC for 90 minutes, 200µl cells were plated and spread on Luria broth (LB) or brain-heart infusion (BHI) agar plates (containing 100µg/ml Ampicillin (50µg/ml)/X-gal (60µg/ml)/IPTG (0.1mM)), either neat or at a 1:100 dilution. BHI plates were used in preference to LB as it provides a more nutrient-rich medium for cell growth.

When high throughput transformations were required, chemically-competent cells provided a cost-effective option. Bioline Alpha-Select Silver Efficiency competent *E.coli* cells were used, again using the standard protocol. However, the purification of the ligation mixture through ethanol precipitation was not generally required and found not to improve efficiency noticeably. After incubation for 90 minutes at 37ºC, the *E. coli* cells were centrifuged at 5000 rpm to pellet the cells and 700µl of supernatant removed. Cells were then resuspended in the medium and 200µl was plated on a BHI agar plate. It was found that concentrating the cells substantially increased the number of clones per plate, compensating for the lower efficiency of the chemically-competent cells, relative to the electro-competent cells.

2.7.3 Clone selection and plasmid extraction for sequencing

The blue-white cloning technique relies on the disruption of the *lacZ* gene in the *lac* operon in the T-easy plasmid vector, and successful disruption inhibits the production of the enzyme β-galactosidase. When β-galactosidase is expressed in the presence of the chemical X-gal, it cleaves X-gal molecules resulting in a blue pigment, which gives colonies of *E.coli* containing vectors containing the *lac* operon a blue colour. Therefore, clones containing inserts are usually apparent due to their white colour. After overnight incubation at 37ºC and assuming a) successful transformation and b) sufficient resolution of cells on the plate, white colonies were picked and smeared on a gridded agar plate (Ampicillin/X-gal/IPTG) with a 200µl pipette tip. These colonies were grown overnight at 37ºC and then checked to see that no blue colour was apparent in the colonies. Presence of the target insert could be ascertained by picking a few cells from each colony and dipping in 100µl of ultra-pure water and using 1µl of this dilution as a PCR template. A PCR was then run on a thermo-cycler using a truncated version of the original parameters (a full length programme usually resulted in over-amplification). Product was run on an agarose (1%) gel using either a ladder or the original PCR product to confirm an insert of the correct size was present. White colonies were then picked and dipped in 5ml of

LB/BHI media with a 10µl pipette tip and incubated in a shaker overnight at 37ºC. Plasmids were extracted from the resulting cultures using Favorgen Favorprep Plasmid Minipreps using the standard protocol (see Appendix ix) and sent off for sequencing by Macrogen Inc. (Korea) specifying M13 universal primers to be used (T-easy vector contains M13 restriction sites either side of the insert location).

Chapter 3 – Microsatellite discovery and characterisation

Second-generation sequencing technology was used to identify short-tandem repeats (microsatellites) in the NSB genome. Polymorphic loci were identified by PCR amplification of DNA from individual birds. A large number of di-, tri- and tetra-nucleotide loci were discovered and, of these, a panel of 10 polymorphic tetra-nucleotide loci were characterised for subsequent population studies. All short tandem repeat loci identified in this investigation were ranked in order of relative frequency of the repeat motifs observed in this species; the results were compared with those previously reported by Primmer *et al.* (1997). The use of second-generation sequencing to identify polymorphic loci for conservation studies of native species proved to be efficient and cost effective and the accumulated data set provides opportunities for future studies involving functional genetic loci. Selected polymorphic loci will be used to estimate genetic diversity and inbreeding levels in the three main NSB subpopulations available for study in this project.

3.1 Introduction

Microsatellites are short-tandem repeating elements of DNA sequence that are found throughout most eukaryotic genomes. These repeating elements are often polymorphic. It is the polymorphic nature of microsatellites that makes them a useful genetic tool for the analysis and evaluation of genetic diversity and they have been applied to describing the genetics of a wide range of taxa (Goldstein & Schlötterer, 1999; Garza & Williamson, 2001; Ellegren, 2004). Generally, microsatellites are designated to be neutral markers as they mostly reside in non-coding regions (Hancock, 1995) and, therefore, are considered not to be under selective constraints. Therefore, theoretically, the extent of polymorphism found in microsatellites regions will be proportional to relative mutation rates within the genome (Ellegren, 2004). The work by Aparicio *et al.* (2007) appears to confirm this assumption, although some studies have questioned the validity of microsatellites as a good indicator of genome-wide diversity (DeWoody & DeWoody, 2005; Väli *et al.*, 2008). However, as a method of establishing a baseline genetic diversity within a species, or even at a

population level, microsatellites can indeed be highly useful, although Hedrick (2001) and Hansson & Westerberg (2002) emphasise the need for caution when interpreting results. Tetra-nucleotide repeating microsatellites are generally preferable to the other smaller di- or tri-nucleotide repeat containing microsatellites as these smaller repeat-motifs tend to be more prone to artefactual stuttering errors (or secondary bands) created during the PCR process (Queller *et al.*, 1993; Whitehouse, 2009). Primmer *et al.* (1997) showed that there is a relative paucity of microsatellites in avian genomes when compared with other taxa e.g. mammals. Furthermore, tetra-nucelotide microsatellites had even greater paucity (Primmer *et al.*, 1997). This potentially presents a real technical difficulty for the discovery of microsatellite loci in birds, particularly when using shotgun cloning methods. A method to overcome some of these failings involves enriching for the motif prior to detection by hybridisation with a radioactive probe (Glenn & Schable, 2005).

This enriched hybridisation technique involves the construction of a genomic library that has been previously enriched for the selected microsatellite motif from which clones are subsequently screened and sequenced (Mindell, 1997; Munyard *et al.*, 2009). This enriched hybridisation technique is both relatively time-consuming and expensive (Saarinen & Austin, 2010). However, 'second-generation sequencing' (SGS) techniques provide a large scale sequencing method to sequence random portions of a species' genome from which large numbers of markers such as micro- and minisatellites can be identified and characterised (Castoe *et al.*, 2010; Csencsics *et al.*, 2010; Saarinen & Austin, 2010; Rotheray *et al.*, 2011; Wang *et al.*, 2012).

This chapter describes the use of 454 Life Sciences pyrosequencing (Roche) SGS to identify microsatellite loci within the NSB genome. Potential target sequences were computationally screened and then used for primer design followed by *in vitro* amplification of NSB template DNA. The aim was to characterise tetra-nucleotide microsatellite loci within the NSB genome and to provide a set of polymorphic loci that could be used in subsequent analyses of the genetic diversity in the three major subpopulations of NSB.

3.2 Methodology

3.2.1 DNA extraction and preparation for 454

Genomic DNA (gDNA) was extracted from blood tissue from an individual NSB (01M04 – a male from MG) using QIAGEN DNeasy Blood and Tissue Miniprep kits as described in 2.3.1. Tissue from 01M04 was used for two main reasons: a) it was sampled from the Two Peoples Bay subpopulation, which is thought to have the highest genetic diversity since it was the source for all other extant subpopulations; b) it was the most recent tissue sample available from this subpopulation. To maximise the yield of gDNA, the tissue sample was divided into two separate samples, extracted using QIAGEN columns and the first and second elution from each extraction kept.

Elutions of gDNA were run on a 1% agarose gel to assess levels of DNA fragmentation. The eluted DNA had some fragmentation but still contained a considerable quantity of high molecular weight DNA. This observed fragmentation was most likely due to the age of the tissue sample (collected in May 2001, ~9.5 years prior to DNA extraction) but was not considered a serious issue as a gel purification of DNA after nebulisation was performed prior to pyrosequencing. Samples were initially concentrated to ~50µl by evaporation in a 60ºC oven for 45-60 minutes. A NanoDrop (Thermo Fisher Scientific) was used to quantify DNA concentrations to ensure samples were adequate for sequencing purposes. Macrogen Inc. stipulate concentrations of >50ng/µl and an overall DNA quantity of >500ng for High Molecular Weight (>1.5kb) samples for 454 sequencing and these parameters were used for appraising sample quality. The sample with the highest concentration and purity (based on A260/A280) was used for sequencing.

3.2.2 Construction of gDNA library using 454 shotgun pyrosequencing

DNA sequencing was performed at the Lotterywest Biomedical Genomics Facility at Royal Perth Hospital on a 454 Life Sciences GS FLX (Roche). Prior to sequencing, the gDNA was fragmented by nebulisation and gel purified to remove smaller fragments from the sequencing process, which was important given the slight level of fragmentation observed after DNA extraction (3.2.1).

454 pyrosequencing uses 28nm beads to which amplicons are bound through emulsion-PCR. Aqueous microdroplets were enveloped by oil to facilitate

independent PCRs resulting in a library of identical sets of template molecules bound to each bead (Shendure & Ji, 2008; Glenn, 2011). These beads were then loaded onto a plate containing 3.5 million picolitre-scale wells. The pyrosequencing-cycle procedure requires the addition of a different nucleotide (adenine, guanine, cytosine or thymine) preceding the addition of the bioluminescent compound luciferin and adenosine 5'-phosphosulphate. When polymerase-driven incorporation of the nucleotide occurs, pyrophosphate is released and in conjunction with ATP-sulphurylase and luciferase a flash of light is emitted which is detected by a corresponding fibre-optic bundle. Flash-detection from multiple cycles determines an incorporation pattern, which can be interpreted as the base-sequence from the template attached to the bead (Shendure & Ji, 2008).

3.2.3 Screening and interrogation of 454 data for microsatellite loci

For the reasons discussed in 3.1, tetra-nucleotide microsatellites are preferable for the work undertaken in this study and consequently tetra-nucleotide motifs were screened for in preference to other short tandem repeats (e.g. di- and tri-nucleotide). Primmer *et al.* (1997) tested a range of tetra-nucleotide motifs for relative abundance in an array of avian genomes across a broad range of taxa and observed that the tetranucleotide motifs AAAC, AAAT, AAAG, AGGG, AAGG, ACAG, ACGG and ACCT were found in the highest frequency. Therefore, these motifs were a logical starting point for screening for suitable microsatellite target sequences.

Furthermore, MSATcommander can be set to identify the location of a tandem-repeat within the sequence read, i.e. the base-number where the microsatellite starts and ends within the read. For a flanking region to be a suitable size for efficient primer design, it was considered that it must be at least >50bp in length. This size allows for the exclusion of sections of the flanking region that may have structural issues (e.g. hairpin loops, self-complementarity etc) or sections containing an excess or deficiency of guanine (G) or cytosine (C), that could result in primer instability during PCR. This parameter was used to identify potential microsatellite sequences containing suitably-sized flanking regions.

Additionally, data for the abundance of di-, tri- and tetra-nucleotide motifs were extracted using MSATcommander, using a minimum 5 repeats for tetra-nucleotide microsatellites and a minimum 8 repeats for di- and tri-nucleotide motifs. Abundance of individual motifs was then quantified using Excel (Microsoft) to interrogate the

dataset. These data could then be compared with other studies of the abundance of short-tandem repeats in the avian genome (i.e. Primmer *et al.*, 1997; Longmire *et al.*, 1999).

3.2.4 Primer design and detection of polymorphic microsatellite loci

Whilst MSATcommander is also capable of designing primers, the primer design software PRIMER3 version 0.4.0 (Rozen & Skaletsky, 2000) was used to manually design primers. This gave more flexibility in the design parameters of each primer set and identify any issues with flanking regions. Details on primer design using PRIMER3 are given in 2.5.1. The identified primer sets were tested for structural problems, and ordered from GENEWORKS (Adelaide, Australia) for subsequent testing by PCR.

24 primer sets were initially tested using PCR amplification of NSB gDNA obtained from eight different individuals representing different subpopulations, including the animal 01M04 as a control. The PCR protocol used is described in section 2.4.1. Optimisation of the PCR conditions required the adjustment of the annealing temperature in the thermo-cycler parameters using increments of 1ºC.

3.2.5 Fragment analysis of polymorphic microsatellites

Forward primers of primer sets shown to amplify polymorphic microsatellites were ordered with Well-RED fluorescent dye labels (Sigma-Aldrich). Only 0.25µM of labelled forward primer was used together with 0.5µM non-labelled reverse primers and PCR performed using the optimal parameters determined for each locus in section 3.2.4 above. Fragment analysis was performed using capillary electrophoresis on a CEQ 8000 (Beckman Coulter, USA) analyser using the Frag-4 program and protocol discussed in section 2.6. Signal saturation was avoided by only using 10ng of DNA template. Results were visually viewed using the integrated CEQ 8000 software package and entered manually into an Excel (Microsoft) spreadsheet.

3.3 Results

3.3.1 DNA extraction and second-generation sequencing

Using the methodology outlined in 3.2.1, three samples of gDNA were extracted and concentrated. Concentrations were measured on a NanoDrop to assess the respective

quantity and purity of the samples. Table 3.1 shows the relative concentrations of gDNA in each of three samples.

The concentration of gDNA was 348 to 385ng/µl. The A260/A280 ratios were between 1.8 and 2.0. These three samples were deemed to be suitable for 454 shotgun sequencing, based on concentration and purity parameters outlined in 3.2.1.

As observed in 3.2.1, there was a degree of fragmentation in each of the three

Table 3.1 Results from NanoDrop DNA quantification of elution samples of gDNA from blood tissue sample from Noisy Scrub-bird 01M04

Sample ID	Tube/Elution No.	Volume (µl)	Conc. (ng/µl)
01m04t1e1	1/1	~50	385.05
01m04t1e2	1/2	~50	249.00
01m04t2e1	2/1	~50	348.68

Conc. concentration of gDNA;

samples. However, given the apparently high concentration and purity of the samples, there was confidence that the extractions of gDNA would still yield a high quantity of sequence reads of suitable length for identification and characterisation of microsatellite loci.

The sample 01M04t1e1 was used for sequencing as it had the highest overall concentration of gDNA. The pyrosequencing run for 01M04t1e1 produced ~301 megabases of raw data comprising 1,090,451 reads (provided in FASTA format) with an average read length of 278 bp, which is lower than the expected average read length. Furthermore, single-strand breaks in the larger fragments of DNA from gel purified prior to pyrosequencing can reduce the average length of the fragment library obtained (R. Allcock *pers. comm.*). However, the size and quantity of reads was expected to still be more than adequate for identification of short-tandem repeats such as microsatellites.

3.3.2 Comparison of microsatellite motif abundances in different studies

The ~1.1 million FASTA reads obtained from the 454 GS-FLX (Roche) were initially screened using MSATcommander version 0.8.2 (Faircloth, 2008). Parameters were set for >4 repeats per microsatellite for tetra-nucleotide repeats, but

di-nucleotide and tri-nucleotide repeats (≥8 repeats) were also screened for comparison. 1,450 tetra-nucleotide microsatellites with five or more repeating elements were identified, along with 2,625 di-nucleotide and 989 tri-nucleotide microsatellites with 8 or more repeats, using the data generated from the 454 sequencing in conjunction with MSATcommander. The abundance of motifs of each of these types of microsatellites were compared between this study, a general study of short-tandem repeats in avian genomes by Primmer's laboratory (Primmer *et al.*, 1997), and in similar study of microsatellite motif abundance in another passerine – the brown-headed cowbird *Molothrus ater* (Longmire *et al.*, 1999). Further analysis of the tetra-nucleotide microsatellites found by MSATcommander showed that the most abundant tetra motifs were ACGG, ATCC and AAGC (and their reverse complements). Nine motifs could be regarded as 'rare', with fewer than 10 microsatellites observed in nearly 1.1 million reads. Figure 3.1 shows the relative abundances of all tetra-nucleotide motifs identified and includes the seven most abundant tetra-nucleotides identified by Primmer *et al.* (1997), and Table 3.2 shows the rank of abundance of these seven motifs compared to that observed in this study. Table 3.3 compares abundance rankings of di- and tri-nucleotide motifs between this study, Primmer *et al.* (1997) and Longmire *et al.* (1999). Figure 3.2 shows the relative abundances of di-nucleotide motifs in this study and Figure 3.3 shows the relative abundances of tri-nucleotide motifs and includes the five most abundant motifs identified by Primmer *et al.* (1997).

Table 3.2 Comparison of abundance of short tandem-repeats of tetra-nucleotide microsatellites in this study and a general study of avian genomes (Primmer *et al.*, 1997). Nucleotide motifs are shown in bold font and the reverse complement sequence shown in parathenses.

Motif (reverse complement)	No. microsatellites (this study)	Abundance rank #	
		This study	Primmer et al. 1997
ATCC (GGAT)	188	2	n/a
AAGG (CCTT)	97	6	3=
AAAT (ATTT)	76	9	1=
AGAT (ATCT)	51	12	n/a
AGGG (CCCT)	7	17	2=
ACAG (CTGT)	3	25=	3=
AGCG (CGCT)	3	25=	n/a

Table 3.3 Comparison of abundance of short tandem-repeats of di- and tri- nucleotide motifs in general study of avian genomes (Primmer *et al.*, 1997), *Molothrus ater* (Longmire *et al.*, 1999) and *Atrichornis clamosus* (this study). Nucleotide motifs are shown in bold font and the reverse complement sequence shown in parathenses.

Motif	No. microsatellites (this study)	Abundance Rank #		
		This study	Primmer et al. 1997	Longmire et al. 1999
AC (GT)	1253	1	1	1
AT	810	2	2	3
AG (CT)	535	3	3	2
CG	27	4	n/a	4
AGC (GCT)	394	1	4	1
AGG (CCT)	180	2	1	2
AAT (ATT)	144	3	2	3
AAG (CTT)	110	4	5	8
CCG (CGG)	69	5	3	5
ATC (GAT)	37	6	n/a	5
AAC (GTT)	28	7	8	4
ACG (CGT)	16	8	6	10
ACC (GGT)	9	9	7	7
AGT (ACT)	1	10	n/a	9

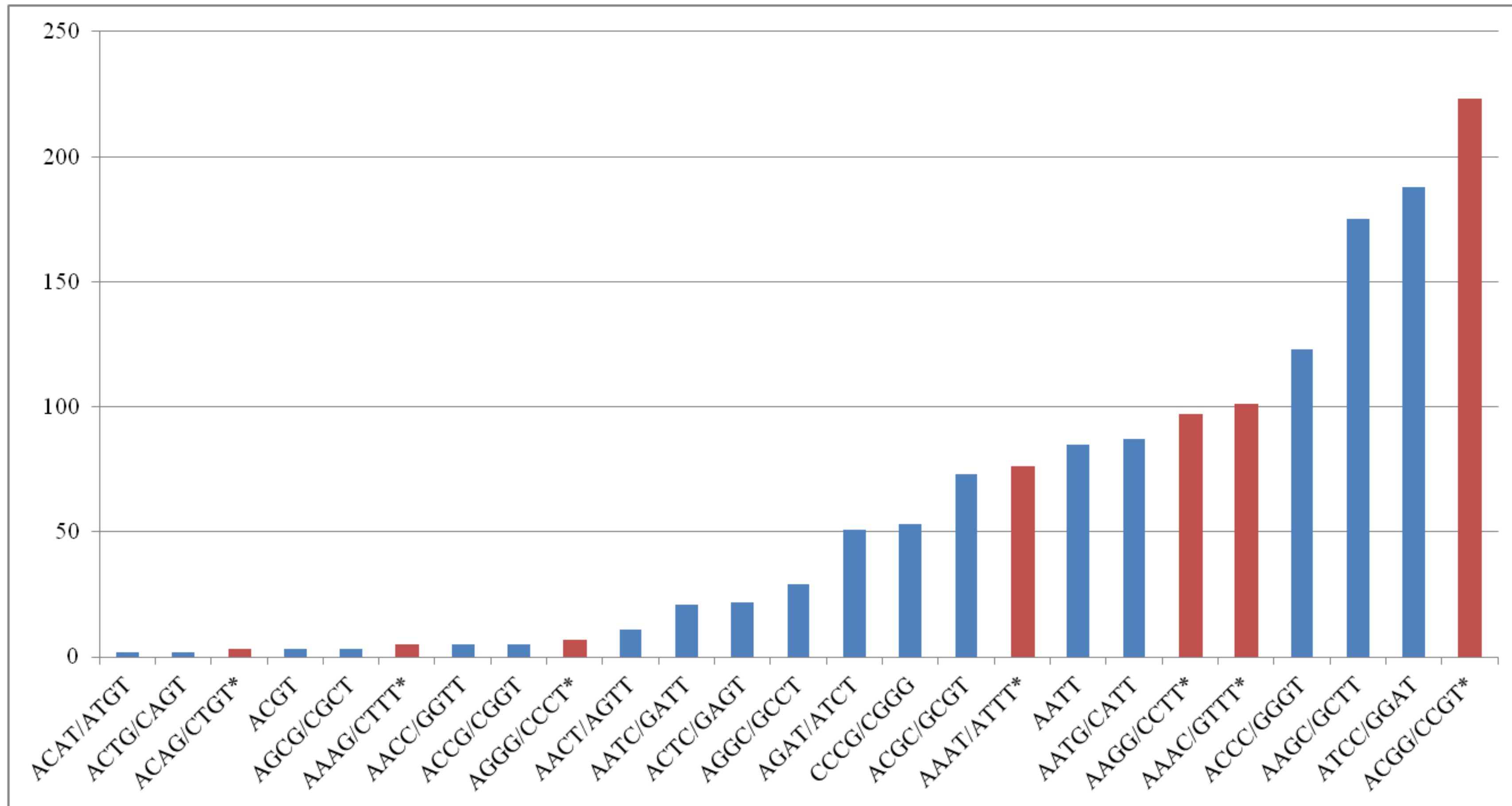


Figure 3.1 Histogram showing the number of individual tetra-nucleotide microsatellites (with 5 or more repeats) observed in the noisy scrub-bird (*Atrichornis clamosus*) 01M04 from interrogated using MSATcommander version 0.8.2 (Faircloth, 2008). The seven red coloured tetra-nucleotides are those observed most commonly across a range of avian genomes by Primmer *et al.*, (1997).

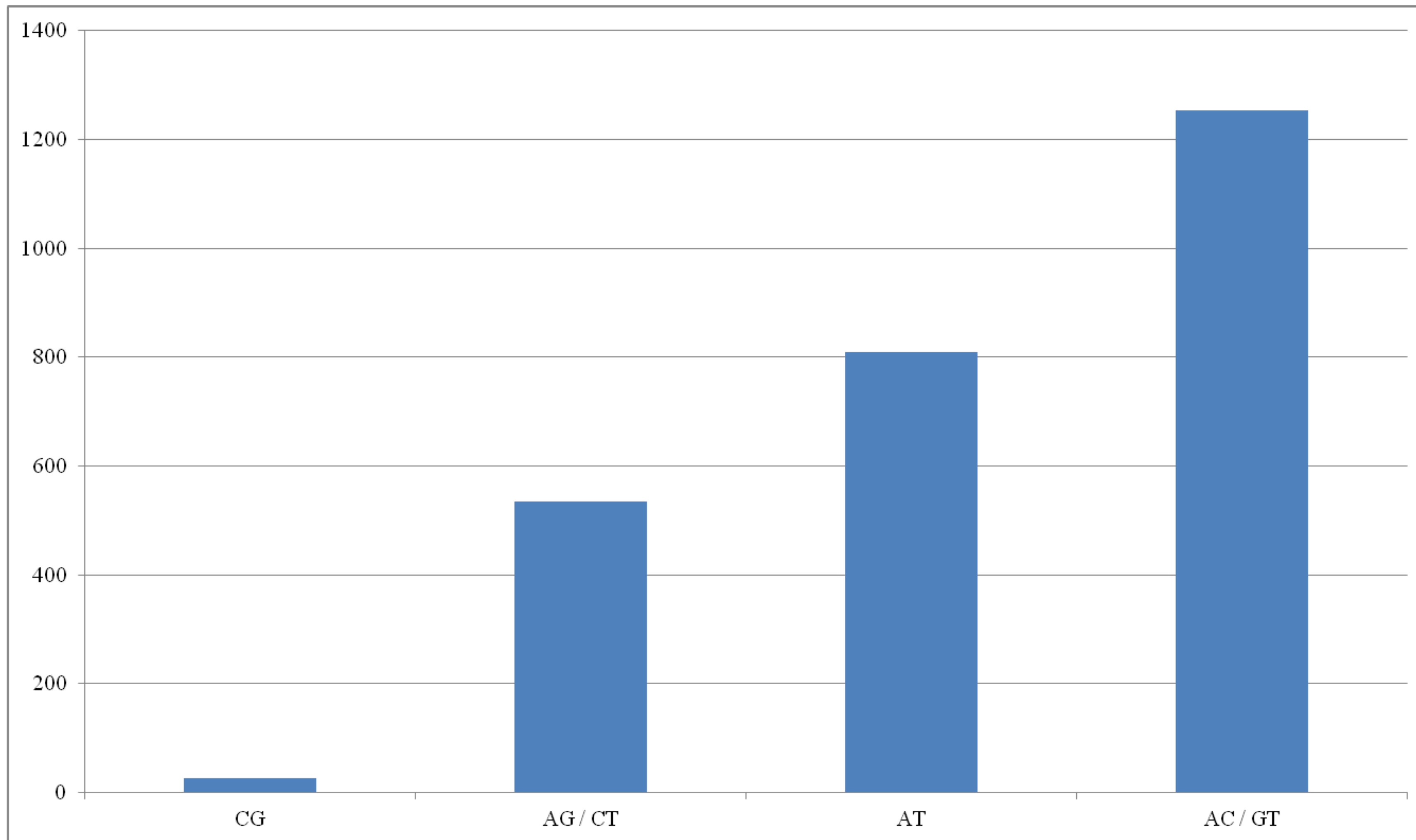


Figure 3.2 Histogram showing the number of individual di-nucleotide microsatellites (with 8 or more repeats) from sequence reads from noisy scrub-bird (*Atrichornis clamosus*) 01M04 from sequences interrogated using MSATcommander version 0.8.2 (Faircloth, 2008).

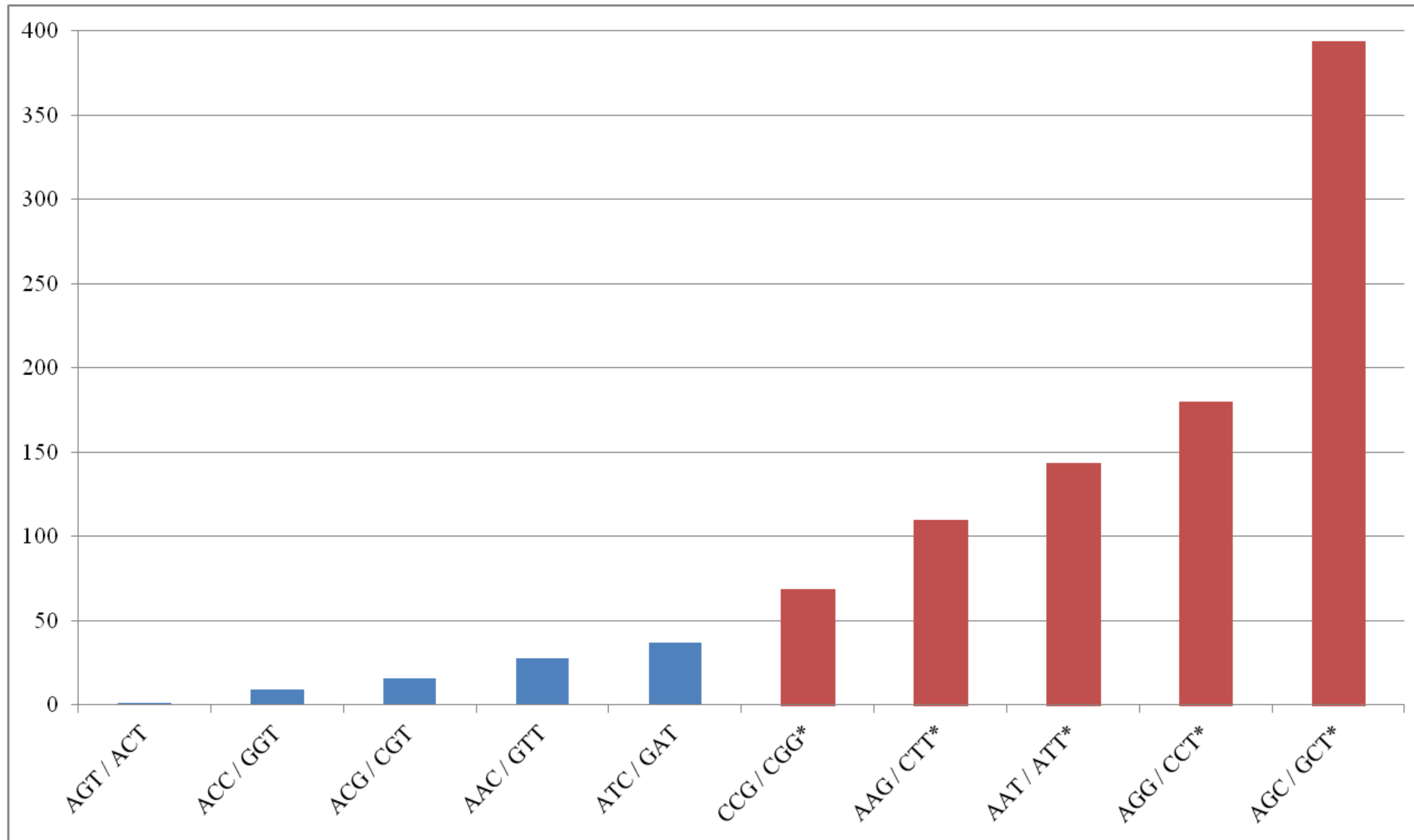


Figure 3.3 Histogram showing the number of individual di-nucleotide microsatellites (with 8 or more repeats) from sequence reads from noisy scrub-bird (*Atrichornis clamosus*) 01M04 from sequences interrogated using MSATcommander version 0.8.2 (Faircloth, 2008). The five red coloured tri-nucleotides are those observed most commonly across a range of avian genomes by Primmer *et al.*, (1997).

3.3.3 *Characterisation of tetra-nucleotide microsatellite loci*

Of the 24 tetra-nucleotide microsatellites chosen as described in section 3.2.3 above, three were shown to be monomorphic, two failed to amplify and three indicated multiple bands (>2 bands) despite repeated attempts to optimise the PCR parameters. The remaining 11 primer sets are shown in Table 3.4 and were optimised for subsequent fragment analysis. A BLAST comparison of the sequences with the NCBI Nucleotide Database (Altschul *et al.*, 2007) (http://blast.ncbi.nlm.nih.gov/) found few matches, although a strong (98% identity) match was found between the lysyl oxidase 4 (LOXL4) gene on chromosome 10 of the chicken (*Gallus gallus*) genome and NSBSC13.

Fragment analysis data was analysed using the CEQ 8000 software and allowed any potential errors from stutter or other PCR- or electrophoresis-related anomalies to be readily identified and samples assigned for repeat testing. One locus (NSBSC18) appeared to be polymorphic but all individuals tested were heterozygous and identical for the same two alleles, which suggests that the presumed "polymorphism" at this locus is more likely the result of non-specific binding or mispriming. This locus was excluded from subsequent from further analysis and its sequence not submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) with the other 10 polymorphic loci (Table 3.4).

Table 3.4 Loci, repeat motifs and associated primer sequences for polymorphic tetra-nucleotide microsatellites observed in an individual (No. 01M04) noisy scrub-bird (*Atrichornis clamosus*)

Locus	GenBank Accession No.	Repeat Motif	Primer sequence (5' to 3')
NSBSC1	KC355442	$(GACA)_9$	F - ACACAGCTTCCTTCCAAACAA
			R - GTGTGCGAGCTTGGCTGAT
NSBSC7	KC355443	$(GACA)_7$	F - CAGCAAGTCCACAAGGGTTT
			R - AAGGTGCTAGGGGCTGATGT
NSBSC8	KC355444	$(AGGG)_7$	F - ACAGGAGCACCTCCAGCTC
			R - CTGCTCACCAGATGCTTCAA
NSBSC11	KC355445	$(TAAA)_5$	F - GCTTTGCAAAACACCCATTT
			R - TTCCTTCCTGTGGGTACCTG
NSBSC13	KC355446	$(AGGG)_6$	F - TCCCGGGAATTTAATCCTGT
			R - GTCTGTCCATCCCATCCATC

NSBSC14	KC355447	$(AGAT)_6$	F - CCAGACAGGGACACCAACTC
			R - ACGGGCAGTAGTGTGAGACC
NSBSC17	KC355448	$(GGAT)_{10}$	F - CAAACTCAGTGCATGAGGAGA
			R - CCATAAATTGCTAAAATACACCATTG
NSBSC18	n/a	$(ATCT)_8$	F - CAAGCAAAACCAGGAGCCTA
			R - TGGTGTTTGTTCAGCCTCTG
NSBSC19	KC355449	$(GGAT)_8$	F - TGCTCCTGACTCTTCCTTCC
			R - TGTCCGATTCCGTCCATC
NSBSC21	KC355450	$(ATCC)_9$	F - TTCAGATAGACCCGGAGAGC
			R - GCACATCACTGCCTGAAATC
NSBSC22	KC355451	$(ATCC)_{10}$	F - TGCAGTGGGTGTGTAAGCTC
			R - AACAAATCAGGAGCCTGTGG

3.4 Discussion

Traditionally, the discovery and characterisation of microsatellite loci has required the use of time-consuming and costly methods such as enriched hybridisation (Saarinen & Austin, 2010). More recently, as supported by the literature, microsatellites are being discovered using more economical methods covered by the umbrella of 'next-generation sequencing' (Shendure & Ji, 2008), as a much more cost-effective alternative to other cloning-based methods (e.g. Castoe *et al.*, 2010; Wang *et al.*, 2012 etc). Pyrosequencing techniques were used in this study as employed by Life Sciences 454 Genome-Sequencer FLX (Roche), which uses relatively small amounts of gDNA and combined with longer read lengths yields literally millions of sequence reads. The relatively long read lengths and high number of reads is particularly useful in studies involving identification of bird microsatellites due to the apparent paucity of microsatellites in avian genomes (Primmer *et al.*, 1997). In this study, generated sequences were specifically interrogated for microsatellite sequences but the datasets generated from SGS can also be interrogated for sequences with applications for variety of different types of studies (conservation, evolutionary studies and structural studies). Furthermore, an analysis of the abundance of di-, tri- and tetra-nucleotide motifs from the SGS data was performed, compared to abundances observed in other studies of microsatellites in avian genomes.

Previous studies have shown that tetra-nucleotide microsatellites are a more reliable tool than di- or tri-nucleotide microsatellites (Eckert *et al.*, 2002) and these were found in relative abundance (with a minimum of eight repeats) in the genome of the individual NSB 01M04. Theoretically, four di-, 10 tri- and 33 tetra-nucleotide motifs could be found in short tandem repeats (Jin *et al.*, 1994) and this study found all possible di- and tri-nucleotide motifs and 25 tetra-nucleotide motifs. Interestingly, several of the tetra-nucleotide motifs shown to be generally abundant across other avian genomes (Primmer *et al.*, 1997) appeared to be rare or even non-existent in the sequences obtained from the NSB genome, while others were found to be relatively common. Of the eight most common tetranucleotide motifs identified in avian genomes by Primmer *et al.* (1997), only three (ACGG (rank 1/27), AAAC (5/27), AAGG (6/27)) were contained within the eight most common motifs in this study, and three were among the rarest motifs (AGGG (19/27), AAAG (22/27), ACAG (25/27) with a fourth (ACCT) absent in this dataset. Primmer *et al.* (1997) analysed a limited number of motifs in their study, the data from this study shows that there is substantial variation in motif abundance in different taxa. Furthermore, in a general study of microsatellite motifs, the most abundant tetra-nucleotide motifs (AGAT, ACAG, AAAT) in non-mammalian vertebrates were not among the most abundant in this study (Tóth *et al.*, 2000).

By contrast, the pattern of abundance rankings of di- and tri-nucleotide motifs in this study was more similar to that shown by Primmer *et al.* (1997). As reported for vertebrates in general by Tóth *et al.* (2000), tetra-nucleotide repeats outnumbered tri-nucleotides in this study. The rank order of di-nucleotide motifs in this study was identical to Primmer *et al.* (1997) and while the rank order of tri-nucleotide motifs differs somewhat, the top five motifs are the same for both studies (although CG appears to be quite rare) the most abundant tri-nucleotide motif (AGC/GCT) is more than twice as common as the next most abundant. However, while AGC/GCT is considerably more abundant that any other tri-nucleotide motif in this study, this motif is considerably less abundant in most taxa (including other bird species) (Meglécz *et al.*, 2012) and its apparent profusion in the NSB genome appears to be quite unusual. In the brown-headed cowbird, another species in the order Passeriformes, four of the five most abundant tri-nucleotide motifs were the same as in this study (Longmire *et al.*, 1999) and although the ranking of di-nucleotide motifs differs slightly from this study and Primmer *et al.* (1997), the AC/GT motif is the

most abundant in all three studies. Therefore, while tetra-nucleotide motifs in the NSB may differ in abundance from the work done by Primmer *et al.* (1997), the di- and particularly tri-nucleotide motif abundance in the NSB appears to closely resemble that observed in avian genomes in general.
The data from this study also demonstrates how difficult it is to generalise about the frequency of particular motifs across a range of taxa, even across closely related species. Furthermore, this study adds to the body of information regarding the abundance of short tandem repeat motifs in avian genomes. The similarities observed in all three studies for di- and tri-nucleotides, when compared to the differences shown for tetra-nucleotides, suggest that the observed patterns may be related to the evolutionary history of the avian genome. Data presented by Tóth *et al.* (2000), showing taxon-specific motif frequencies, appears to support this.

Of the 1,450 tetra-nucleotide microsatellites identified by MSATcommander, 24 loci were tested and 11 characterised, of which 10 were shown to be polymorphic and hence useful in the subsequent analysis of genetic diversity in the NSB. Proportionally, fewer than 50% of the microsatellites of the loci tested in this study were found to be useful for a study of genetic diversity. However, given the large number of tetra-nucleotide microsatellites identified, clearly there is great potential for a large suite of loci to be characterised. Nevertheless, the 10 loci characterised at present will provide a suitable array with which to perform robust genetic analyses. Of the 13 tetra-nucleotide repeats not characterised, three were found to amplify multiple bands, despite persistent attempts to optimise PCR conditions, which indicates that the sequence in the flanking region (to which the primer set binds) may be found at multiple loci, possibly on multiple chromosomes, rather than non-specific binding. It is unclear whether the fragments amplified in these instances are also short tandem repeats, but it may be that that they are the result of an evolutionary mechanism, such as the migration of a transposable element. The large number of repeats identified from the 454 data clearly demonstrates the utility of SGS as a cost- and time-efficient method of identifying and subsequently characterising microsatellite loci for a study of this nature, especially in comparison to traditional methods such as enriched hybridisation. SGS in this case proved highly successful, in spite of the somewhat fragmented nature of the DNA extracted from the original tissue sample.

The 10 polymorphic microsatellite loci characterised in this chapter were deemed a suitable number based on the fact this was the median number of polymorphic loci used in 27 separate studies of genetic diversity in birds between 1997 and 2012 (see Table 3.5). Furthermore, some of the studies in Table 3.5 were able to make use of loci that had been developed for other taxa, thus reducing the financial and time expenses involved in developing primers for novel microsatellite loci. While more loci may have provided a more robust dataset, the costs involved in characterising more loci were evaluated as been not worth the benefit to the study. In total 24 microsatellite were actually characterised but only 11 were polymorphic, so to have a total of 20 polymorphic loci, it might be necessary to characterise a further 24 or more loci.

Table 3.5 Bird species which have had microsatellite diversity investigated and the number of polymorphic loci characterised for each species

Common Name	Scientific Name	*N*	Reference
Asian crested ibis	*Nipponia nippon*	11	He *et al.*(2006)
Balearic shearwater	*Puffinus mauretanicus*	8	Genovart *et al.* (2012)
California condor	*Gymnogyps californianus*	17	Romanov *et al.* (2009)
eastern bristlebird	*Dasyornis brachypterus*	6	Roberts *et al.* (2011)
Galápagos hawk	*Buteo galapagoensis*	17	Bollmer *et al.* (2011)
golden-cheeked warbler	*Dendroica chrysoparia*	9	Lindsay *et al.* (2008)
greater prairie-chicken	*Tympanuchus cupido*	6	Johnson *et al.* (2009)
greater sage-grouse	*Centrocercus urophasianus*	13	Bush *et al.* (2010)
Hawaiian goose (nene)	*Branta sandvicensis*	8	Veillet *et al.* (2008)
hazel grouse	*Bonasa bonasia*	8	Rutkowski *et al.* (2012)
Krüper's nuthatch	*Sitta krueperi*	8	Albayrak *et al.*(2012)
Laysan finch	*Telespiza cantans*	10	Tarr *et al.*(1998)
little spotted kiwi	*Apteryx owenii*	14	Ramstad *et al.*(2010)
Madagascar fish-eagle	*Haliaeetus vociferoides*	22	Johnson *et al.* (2009)
Mariana crow	*Corvus kubaryi*	9	Tarr & Fleischer (1999)
Mauritius kestrel	*Falco punctatus*	12	Groombridge *et al.* (2000)
Merriam's turkey	*Meleagris gallopavo merriami*	9	Mock *et al.*(2004)
millerbird	*Acrocephalus familiaris*	4	Addison & Diamond (2011)
Rarotonga monarch (kakerori)	*Pomarea dimidiata*	7	Chan *et al.* (2011)
Réunion cuckoo-shrike	*Coracina newtoni*	17	Salmona *et al.*(2012)
saddleback	*Philesturnus c. carunculatus*	7	Taylor & Jamieson (2008)
Seychelles warbler	*Acrocephalus sechellensis*	50	Richardson *et al.* (2000)
Spanish imperial eagle	*Aquila adalberti*	18	Martínez-Cruz *et al.* (2004)
stitchbird (hihi)	*Notiomystis cincta*	19	Brekke *et al.* (2011)
takahe	*Porphyrio hochstetteri*	9	Grueber & Jamieson (2011)
white-fronted chat	*Epthianura albifrons*	18	King *et al.*(2012)
whooping crane	*Grus americana*	13	Glenn *et al.* (1997)

Note: median no. of polymorphic loci = 10

Of the 11 tetra-nucleotide microsatellite loci characterised by this study, BLAST searching with the NCBI database found a strong match for just one locus

(NSBSC13), which was the LOXL4 gene on chromosome 10 of the chicken. This result validates the assertion that the microsatellites characterised in this study are almost entirely found in non-coding regions and as such serve as an indicator of global genetic diversity in regions of the genome not under selection.

In summary, SGS proved an efficient and cost-effective method of producing a large quantity of genomic DNA sequence reads, which were then subsequently successfully used to characterise 10 tetra-nucleotide microsatellite loci. Furthermore, the SGS data were also used to perform a comparative analysis of motif abundance between this study and other cognate studies as well as identifying other sequences from functional genes that can be used in other studies.

Chapter 4 – Microsatellite diversity in three genetic populations of noisy scrub-bird (*Atrichornis clamosus*)

In Chapter 3, the first tetra-nucleotide short-tandem repeats were identified and characterised for the NSB. Here, 10 polymorphic loci were used to carry out an analysis of genetic diversity and inbreeding in 60 individuals from the three main subpopulations of this species: Mt Gardner (n = 22), Mt Manypeaks (n = 23) and Bald Island (n = 15). Several statistical analyses were performed, including departure from Hardy-Weinberg Equilibrium, Wright's F-statistics, linkage disequilibrium and genic differentiation. These data were also used to model putative bottlenecks and intra-population structure. This work represents the first intra- and inter-population analyses of genetic diversity for all major subpopulations of this species and provides a valuable insight into the population history and implications for genetic management in this species.

4.1 Introduction

As discussed in 1.5.2, the NSB has had a turbulent history in modern times. Once spread patchily across the south-west of Western Australia (WA), it was lost to science at the turn of the 20th Century, a mere half century after discovery. Fortunately, it was serendipitously rediscovered in 1961 at what is now Two Peoples Bay Nature Reserve (TPB) near Albany on the south coast of Western Australia (Danks *et al.*, 1996). This population has become the subject of intensive research and management, leading to a steady population increase which was followed by translocations of birds to other areas in the Albany region as well as locations in the Darling Range (where the species was originally discovered in the 19th Century). While some translocations around the Albany area (e.g. Mt Manypeaks, Bald Island) have proved successful, others have been unsuccessful for a variety of reasons, with feral predators and wildfire implicated as factors (Comer *et al.*, 2010).

The potential long-term success of translocations can be improved by maximising the genetic diversity in the newly established population. This can be achieved by

introducing a sufficiently large founder group, for example, Briskie & Mackintosh (2004) recommend a minimum of 150 individuals. In addition, the breeding system may need to be considered, since differences in inter- and intra-gender reproductive success will affect effective population size (N_e) (Nunney, 1993), which in turn will influence the number of individuals contributing to the gene pool of the population. Initial founder groups may need to be consolidated by additional translocations (Allendorf & Luikart, 2007), thereby reducing the 'genetic load' of the population (Ingvarsson, 2001; Hedrick, 2005), that is, the reduction in mean fitness of a population due to mechanisms such as mutation, recombination etc, compared with a population where these mechanisms have not occurred (Keller & Waller, 2002). However, integrating all these considerations into a translocation program is rarely practicable, especially with non-sessile organisms, as time, money, personnel and other resources are often limited. As a result, translocations often use considerably fewer individuals than what is considered ideal (Briskie & Mackintosh, 2004) and this will be more likely in the case in species that are rarely observed or cryptic, such as the NSB. However, downstream evaluations may provide an alternative by informing conservation managers of the long-term viability of a population based on the genetic diversity that has been sustained from artificial founder events such as translocations (Sarrazin & Barbault, 1996; Cardoso *et al.*, 2009; De Barba *et al.*, 2010). This allows any observed loss of genetic diversity to potentially be mitigated by assisted migrations or through additional translocations, known as 'genetic rescue' or 'restoration' (Ingvarson, 2001; Bouzat *et al.*, 2009).

In spite of the success of translocations to Mt Manypeaks and Bald Island, these populations were founded with very small groups, with 32 and 11 individuals released in each location in 1983-1985 and 1992-1993 respectively (Danks *et al.*, 1996). This is much smaller than the figure recommended by Briskie & Mackintosh (2004). Furthermore, no account was made of breeding systems or effective population size, as at the time of translocation little knowledge about either of these two factors (Danks *et al.*, 1996; DPaW, 2014) was available. The dramatic contraction of NSB's distribution and the prolonged nature of its isolation at TPB probably resulted in a significant population bottleneck, similar to that experienced by the South Island saddleback (*Philesturnus carunculatus carunculatus*), where a large-scale contraction led to the reduction from 143 alleles at 22 loci in the

historical mainland population to 35 alleles in the last remaining island population (Taylor *et al.*, 2007). In addition, the introduction of such small founder groups can theoretically impose serial population bottlenecks, which have been shown previously to further compound genetic diversity loss in small populations (Gautschi *et al.*, 2002; Lambert *et al.*, 2005). Since genetic diversity has been shown to have strong links to both extinction risk (Frankham, 2005; Evans & Sheldon, 2008) and fitness (O'Brien *et al.*, 1987; Roelke *et al.*, 1993; Briskie & Mackintosh, 2004; Hale & Briskie, 2007), it is a reasonable assumption that any significant reduction in genetic diversity will be detrimental to the long-term viability of any population, let alone a new population established through artificial means. Therefore, it is possible that low genetic diversity has contributed somewhat to the failure of some translocations e.g. the Darling Range.

However, in contrast to the failed translocations, the Mt Manypeaks and Bald Island translocations have met with meteoric success, with the populations peaking at 427 active male territories in the Mt Manypeaks census zone in 2001 and 114 territories on Bald Island in 2012 (DPaW). The Mt Manypeaks population was subsequently decimated by a wildfire in 2005 resulting in just 60 territories being recorded in 2006 but has recovered rapidly since to 146 territories by 2011 (DPaW). Given that female NSBs will raise a maximum of one chick per breeding season, these figures indicate high levels of recruitment, which in turn should reflect higher fecundity and minimal difficulties with fitness. This paradox between founder group size and apparent population health can be partially resolved for Mt Manypeaks, since there is evidence for some movement of birds from TPB through the Angove Water Reserve to the west (Danks *et al.*, 1996) and it is also likely that there has been gene flow from descendants of the Mermaid translocation to the east in 1992-1994. However, the Bald Island appears to confound this theory since the island is 1-2km from the mainland and the chances of movement of semi-flightless NSBs across this stretch of water are negligible, we can assume that this represents a closed population. Therefore, the remarkable increase from 6 territorial males in 1994 to 114 in 2012 has occurred in isolation from all other populations and while the successful translocation of groups of genetically depauperate birds has precedent (Taylor & Jamieson, 2008) and the predator-free status of Bald Island is likely to be a contributing factor, the scale of the success of this translocation is surprising.

In contrast to the successful establishment of new populations at Mt Manypeaks and Bald Island, the original parent population at TPB has somewhat stagnated. The Mt Gardner population (within TPB) peaked at 179 territories in 1994 then subsequently declined to 111 territories in 2012 (DPaW), while the Lakes population (representing a meta-population sink to Mt Gardner's source) has had no more than 1-2 active territories since 2000 when a wildfire wiped it out. Additionally, the occurrence of what is presumed to be 'rubber-bill' syndrome in a nestling at Mt Gardner, most likely resulting from 'secondary hyperparathyroidism or calcium deficiency' (Tiller *et al.,* 2007), has raised alarm bells about the fitness of this population.

Despite the fact that there has been a significant recovery in the global population of the NSB since its rediscovery in 1961 (Danks *et al.*, 1996; Comer *et al.*, 2010), the fires in the Lakes in 2000 and Mt Manypeaks in 2005 resulted in a decline from 773 to 343 active territories (Burbidge *et al.*, 2005) ultimately leading to the uplisting of the NSB from Vulnerable to Endangered by IUCN (2012) (www.iucnredlist.org). Therefore, the NSB is still of high conservation concern and the need to understand the species' population genetics is a priority to inform the ongoing translocation program (Danks *et al.*, 1996; DPaW, 2014).

To elucidate the genetic profile of the TPB population, as well as the two most significant translocated populations on Mt Manypeaks and Bald Island, an analysis of microsatellite diversity in these populations was undertaken, using the 10 loci previously described in Chapter 3. All three main populations (TPB, Mt Manypeaks and Bald Island) (Figure 4.1) were treated as separate genetic populations (demes) as these populations are effectively islands with little or no gene flow between them. It is predicted that the genetic diversity would be generally low but it is expected that the highest diversity would be found in the parent population at TPB. Lower diversity is also predicted for the Mt Manypeaks and Bald Island populations in descending order, purely based upon the number of founders that established each population. Furthermore, it is predicted that the levels of inbreeding are expected to be the highest in the Bald Island population, as this population resulted from a founder group of just 11 individuals (including just 3 females), and the lowest levels of inbreeding in the TPB population. Finally, it is hypothesised that the highest genic differentiation will occur between the two translocated populations as they result from a small sample of the available genetic variation in the TPB population that

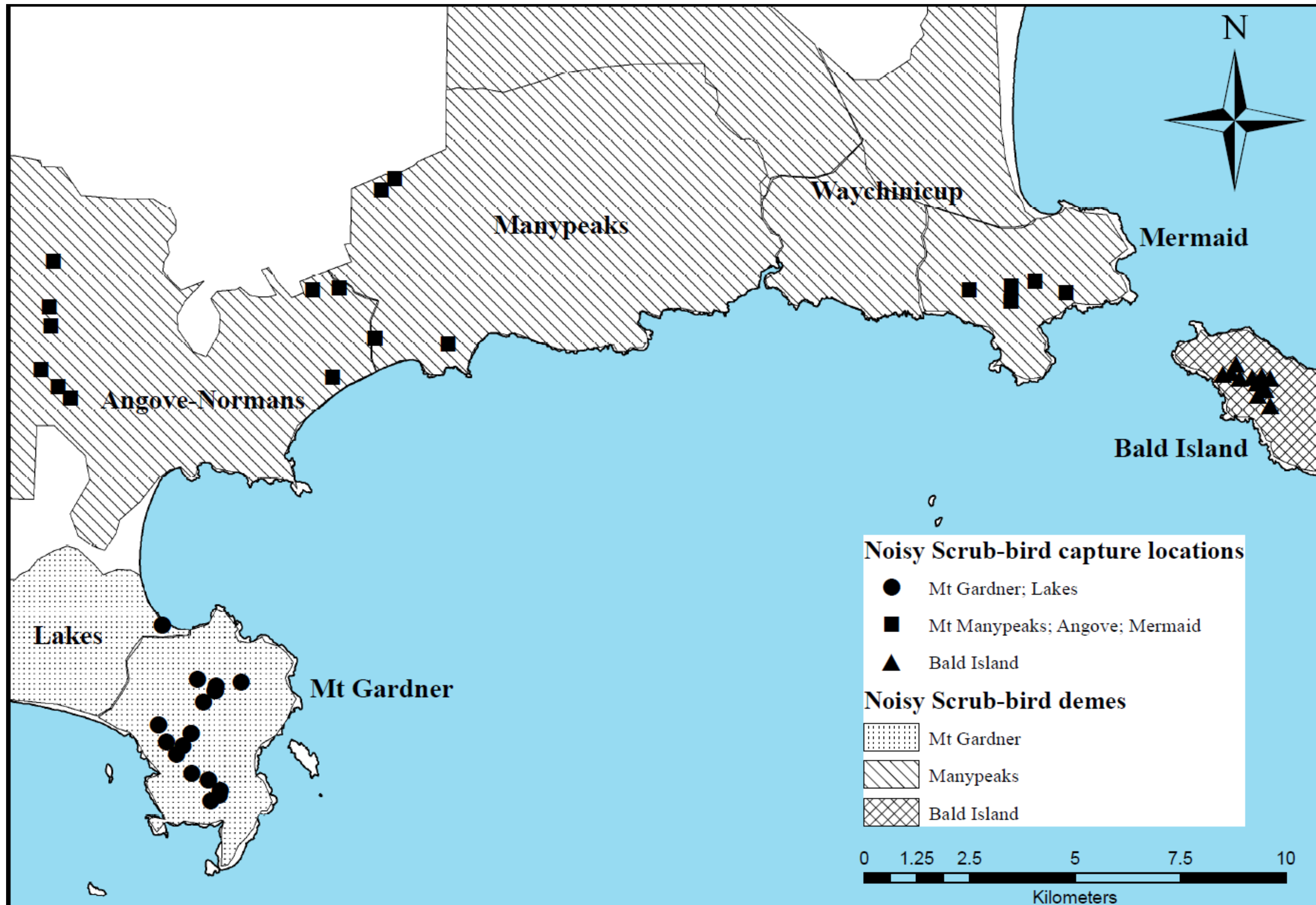


Figure 4.1 Locations of noisy scrub-birds sampled for DNA analysis from all three subject demes in the Albany Management Zone

were acted on by processes such as inbreeding and genetic drift (Keller & Waller, 2002). These data can also be used to try to establish the effective founder population

size (i.e. the number of founders that reproduced) of the Bald Island population, since all genetic variation within this population is derived from this group.

4.2 Methodology

4.2.1 Analysis of microsatellites by capillary electrophoresis

The 10 primer sets for polymorphic microsatellites characterised in Chapter 3 were used to amplify loci by PCR in gDNA samples (2.3 & 2.4) from NSBs captured as part of the ongoing translocation program. A total sample size of 60 individual NSBs were used (see Appendix i), with 22 from TPB, 15 from Bald Island and 23 from the contiguous populations from Angove Water Reserve in the west, through Mt Manypeaks Nature Reserve and Waychinicup National Park to Arpenteur Nature Reserve in the east (Figures 1.6 & 1.8). The latter area was treated as one discrete genetic population or deme (MP), with the two meta-populations of Mt Gardner and Lakes at TPB being treated as another separate deme (MG). Bald Island was designated as a deme by itself (BA), due to the high improbability of gene flow between this population and any mainland population. All three demes represent 'islands' where minimal or negligible gene flow is thought to occur. Temporal variation between samples was high, with MG samples dating from 1998 to 2001, MP from 2000 to 2007 and BA from 2009 to 2011. These temporal differences have been considered when drawing conclusions about contemporary diversity.

The gDNA was extracted from blood using QIAGEN DNeasy Blood and Tissue and AXYGEN AxyPrep Blood DNA Miniprep Kits (2.3.1) (n = 57) and from feathers using XytXtract – Animal (http://www.xytogen.com.au) (Castalanelli *et al.*, 2010) (2.3.2) (n = 3). PCR was carried out using fluorescent-labelled forward primers (Well-RED (Sigma-Aldrich)), which allowed the products to be analysed by capillary electrophoresis on a CEQ 8000 (Beckman Coulter) (2.6). Results were screened manually using the supplied CEQ software and entered into an Excel (Microsoft) spreadsheet.

4.2.2 Statistical analysis of microsatellite data

Analyses of genetic diversity and inbreeding coefficients in all three demes was performed using GENEPOP version 4.0.10 (Raymond & Rousset, 1995). This software calculates deviation from Hardy-Weinberg Equilibrium (HWE)

(heterozygote deficiency/excess), observed vs. expected heterozygosities (H_O vs. H_E), linkage disequilibrium (LD), genic differentiation (Fisher's exact probability test (Raymond & Rousset, 1995) and F-statistics (F_{IS}, F_{ST} and F_{IT}) (Weir & Cockerham, 1984). LD and Departures from HWE (including F-statistics) and were tested for statistical significant ($p < 0.05$) using a Markov-chain algorithm, with 1000 dememorizations, 100 batches and 1000 iterations per batch. This type of statistical test is usually sufficient for accurate estimates but can be biased by large standard errors, which can be corrected by increasing the number of batches. A Bonferroni correction was applied to *p* values to correct for multiple testing. Statistical significance of variation between populations for allelic richness and F_{IS} was calculated using a Wilcoxon signed-rank test.

While Balloux *et al.* (2004) suggested that there is an underlying weakness in the assumption that small numbers of loci can be used to infer inbreeding, (i.e. F-statistics), they also stated that in small populations and populations with skewed mating systems these estimates may be more accurate. Both of these elements are relevant with the NSB, as it has relatively small populations in three hypothetical demes, which have low or no gene flow between them, and a hypothesised polygynous mating system (1.5.1). The potential for 'null alleles' (alleles that exist but are not amplified by PCR due to a mutation at the primer-binding site) exists in any study of this nature and results were screened for 'null alleles' by Microchecker 2.2.3 (van Oosterhout *et al.*, 2004). Mean alleles per locus (MAL) was calculated by dividing the total number of alleles recorded in a population by the number of loci ($n = 10$). However, a more robust estimate of genetic diversity across population is mean allelic richness (MAR) and this was calculated using HP-RARE version 1.1 (Kalinowski, 2005), which alleviates bias caused by uneven sample sizes through the calculation of the rarefaction on private alleles.

To test whether three hypothetical demes (MG, MP & BA) represent structurally different populations, STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000) was used. STRUCTURE uses a Bayesian model computation to estimate clustering in a dataset to infer population structure. STRUCTURE also uses a Markov-Chain Monte-Carlo (MCMC) algorithm to predict the probability of the different structures, with varying values of *K*, (where *K* represents the hypothetical number of populations). Initially a burn-in time of 100,000 repetitions of the MCMC was used before starting data-collection for 500,000 repetitions but it was found that burn-in needed to be

increased to 200,000 with another 2,000,000 repetitions to achieve consistent probability values (as per Hicks *et al.*, 2007). Both admixture and non-admixture models were used: the admixture model presumes that individuals may be of mixed ancestry; the non-admixture model is more powerful at detecting subtle population structure and also assumes discrete populations which will test the hypothesis that the MG, MP and BA populations are discrete demes (Pritchard *et al.*, 2000). However, while testing for genetic structure is valuable, authors have found that inappropriate values of *K* (Kalinowski, 2011) and high LD (Kaeuffer *et al.*, 2007) can cause bias with the modelling of clusters. The latter issue is of less relevance to this study, the key question is whether the three arbitrary subpopulations represent true genetic 'demes', but caution should be exercised if LD values are found to be high.

In addition, spatial autocorrelation within all populations was modelled using GENALEX version 6.5 (Peakall and Smouse 2006, 2012) (using genetic distance vs. geographic distance using the Spatial Structure and Mantel Test analyses) and population assignment was performed for all three populations using the Population Assignment test. The Spatial Structure analysis uses the values for pairwise genetic distance to calculate the autocorrelation coefficient *r* and how it varies with geographic distance, whereas the Mantel Test compares pairwise genetic and geographic distance directly for each individual. The Population Assignment seeks to assign individuals to one of a given number of population based on their genotype.

Another use of microsatellites in genetic diversity studies is the modelling of founder events and recent bottlenecks (Spencer *et al.*, 2000). In this study the microsatellite data used to a) model the putative effective founder size for the BA deme (i.e. the number of individuals that actually reproduced post-translocation), since the exact number of individuals released in this translocation is known and that the possibility of any migration to the island is unfeasibly low; b) demonstrate whether these data can predict bottlenecks for any of the three demes. For the modelling of the effective founder size for Bald Island, the following equation was used (Hedrick, 2005):

$$H_f = H_s\left(1 - \frac{1}{N_f}\right)$$

where H_f is the observed heterozygosity of the founder population (BA), H_s is the observed heterozygosity of the source population (MG) and N_f is the number of effective founders. This has been used by other studies to establish the number of

founders in new populations (e.g. Kalinowski *et al.*, 2010; Alda *et al.*, 2013). Calculations were performed in Excel (Microsoft) and constructed 95% and 90% confidence intervals across loci using PowerMarker version 3.25 (Liu & Muse, 2005).

The presence of bottlenecks was modelled for using BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996), which simulates a coalescent process under an Infinite Allele Model and a Stepwise Mutation Model where the expected heterozygosity of a sample for a given number as loci is calculated, using the assumption of mutation-drift equilibrium. Results of this modelling process will indicate whether the allele-frequency distribution is 'L-shaped' (i.e. what would be expected under mutation-shift equilibrium) or whether there has been a mode shift, indicating a recent bottleneck. While bottleneck tests can be unreliable and overestimate the occurrence of bottlenecks (Peery *et al.*, 2012), this test is included in this study to confirm that the BOTTLENECK software can estimate bottlenecks, using the given dataset, that are known to have occurred (e.g. the introduction to Bald Island), i.e. a test of the data and the software rather than an attempt to uncover past bottlenecks.

Finally, the microsatellite data was used to attempt to model effective population size (N_e) (i.e. the number of breeding individuals in a population (Wright, 1931)) for the MG deme, using the software Ne-ESTIMATOR version 2.01 (Do *et al.*, 2004), which computes N_e using the Linkage Disequilibrium model (Waples & Do, 2010), Heterozygote Excess model (Pudovkin *et al.*, 1996) and Molecular Coancestry method (Nomura, 2008).

Many of these methods use genotypic frequencies and departures from HWE from this to calculate their estimates. Therefore, any errors with these data may bias any subsequent analyses. However, while this is a possibly limitation, algorithms in software packages such as STRUCTURE, Ne-ESTIMATOR and BOTTLENECK are widely used in genetic diversity studies and underline the value of the HWE in elucidating different facets of conservation genetics.

4.3 Results

Across the 10 microsatellite loci a total of 33 alleles were recorded. All 33 alleles were found in the MG sample, with 30 in MP and 29 in BA (Table 4.1). MAR was marginally less than MAL for MG (3.15) but values for BA and MP were essentially

identical. Pairwise variation in allelic richness between all demes was statistically significant ($p < 0.01$). The three alleles found in MG represent private alleles that are either rare or non-existent in the other two demes. Two loci were found to be di-allelic (NSBSC11 & NSBSC19).

Table 4.1 Summary of sample sizes from each subpopulation (deme) and allele diversity parameters

Subpopulation	*n*	Loci	Alleles	MAL	MAR
Mt Gardner (MG)	22	10	33	3.3	3.15
Mt Manypeaks (MP)	23	10	30	3.0	2.99
Bald Island (BA)	15	10	29	2.9	2.90

n, number of individuals sampled from each subpopulation; MAL, mean alleles per locus; MAR, mean allelic richness (Kalinowski, 2005)

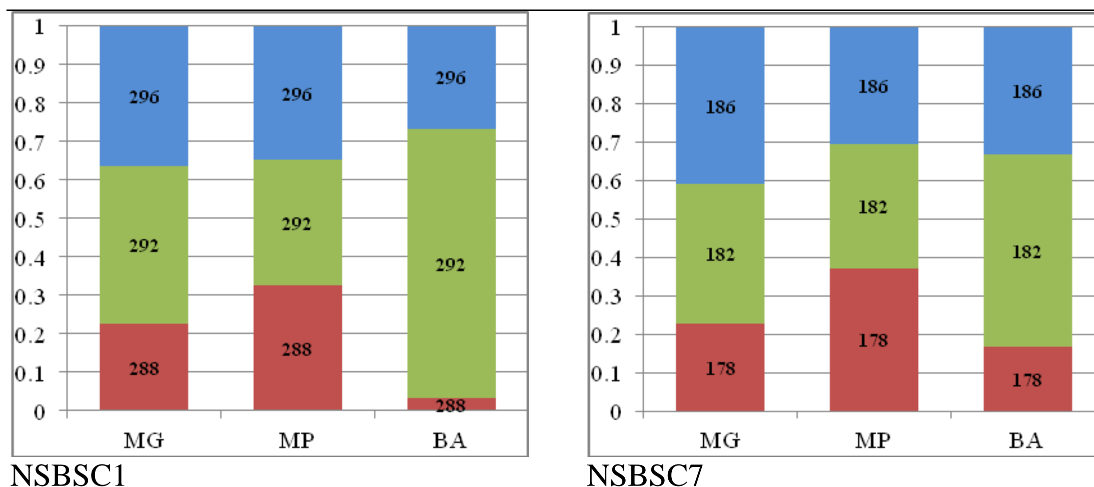
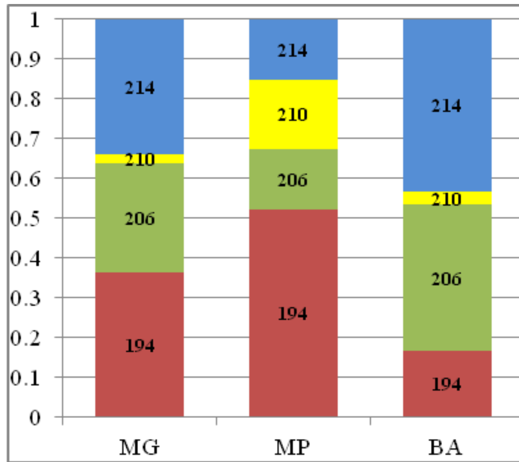
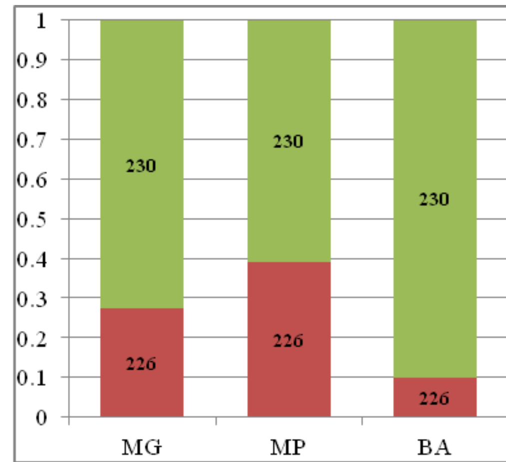


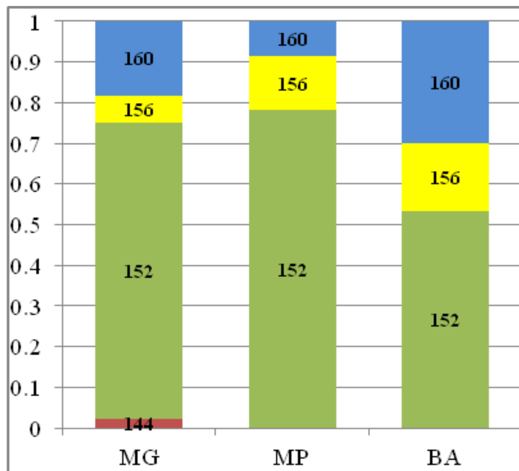
Figure 4.6 Allele frequencies for 10 polymorphic loci in three discrete noisy scrub-bird (*Atrichornis clamosus*) demes (MG *n* = 22; MP *n* = 23; BA *n* = 15) (MG Mt Gardner/Lakes; MP Mt Manypeaks and surrounds; BA Bald Island). Frequencies are given as overall proportion of alleles per locus per deme; allele sizes (bp) displayed in corresponding section of each column.



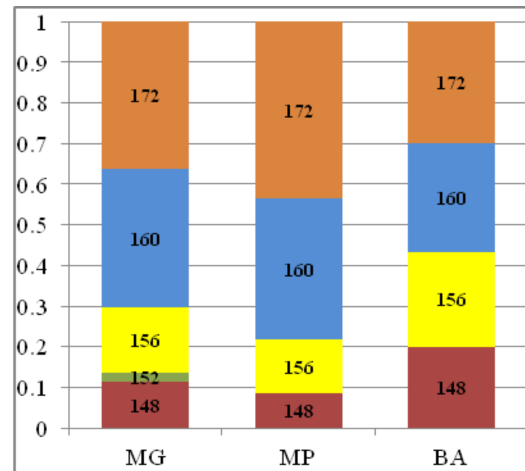
NSBSC8



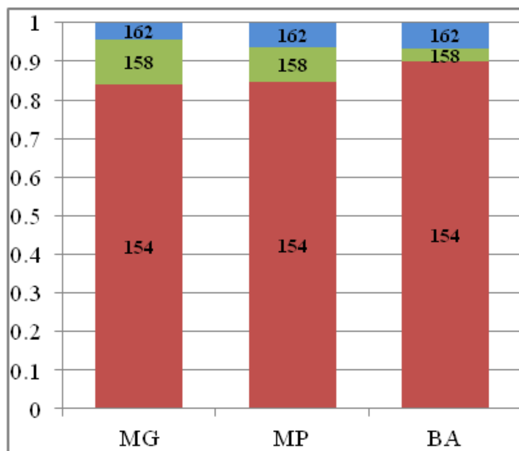
NSBSC11



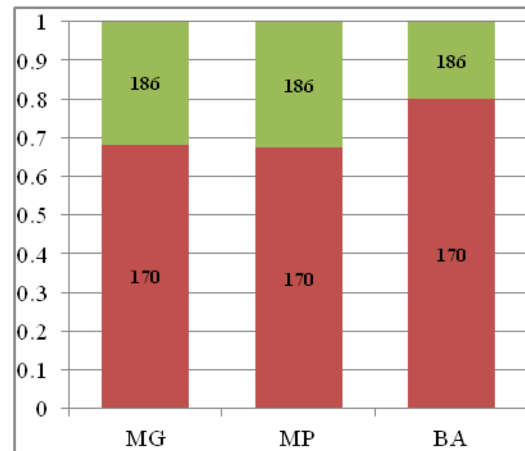
NSBSC13



NSBSC14



NSBSC17



NSBSC19

Figure 4.6 continued

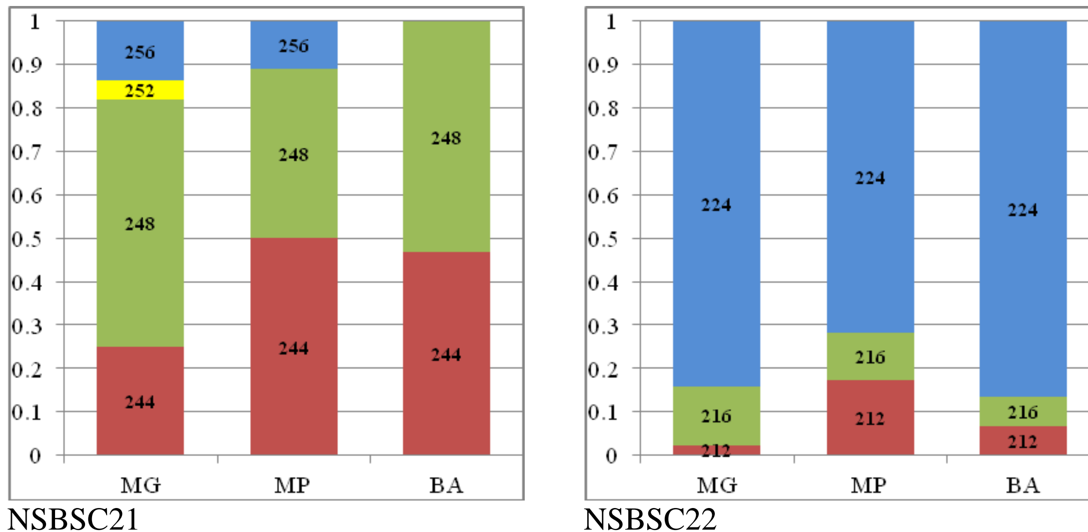


Figure 4.6 continued

Calculations of departure from HWE found significant deviations ($p < 0.05$) for MG and MP but surprisingly not for BA, which appears to be in or close to HWE according to these results. Based on H_O vs. H_E and F_{IS} (inbreeding coefficient) values both MG and MP appear to have a significant excess of homozygotes (Table 4.2), whereas BA also has an apparent homozygote excess, the probability (p) value is not below the 0.05 significance level (including when Bonferroni correction is applied). However, there was found to be no significance between values for F_{IS} (for individual loci) for all three populations. Analysis by Microchecker identified two loci containing putative 'null alleles' (NSBSC1 & NSBSC13) for MG and MP and inconsistently (based on multiple runs) assigned a third (NSBSC19) for MG. However, excess homozygosity such as that observed in MG and MP produces very similar results to those if 'null alleles' are present (van Oosterhout *et al.*, 2004) so it is difficult to assess whether the results produced by Microchecker are accurate or simply an artefact of true homozygote excess in that sample. Calculations of HWE were performed again, excluding the loci NSBSC1 and NSBSC13 both MG and MP (and overall for all populations) no longer showed a significant deviation from HWE (after Bonferroni correction) (Table 4.2). However, the observation that no 'null alleles' were assigned for any loci in BA (which does not significantly deviate from HWE) provides evidence that 'null alleles' are unlikely to be the cause of the observed deviation. Inter-population F_{ST} (fixation index) values were generally low across all loci and the overall figure of 0.025 indicated that as little as 2.5% of the observed variance is due to population subdivision.

Table 4.2 Summary of estimates of heterozygosities and F-statistics (Weir & Cockerham, 1984) for all three demes and for all populations combined

	MG			MP			BA			ALL		
Locus	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}	H_E	H_O	F_{IS}	F_{IS}	F_{ST}	F_{IT}
NSBSC1	0.664	0.364	0.458*	0.681	0.435	0.367*	0.453	0.400	0.120	0.359	0.052	0.392
NSBSC7	0.664	0.545	0.182	0.679	0.652	0.041	0.632	0.667	-0.057	0.070	0.005	0.075
NSBSC8	0.707	0.500	0.283	0.666	0.565	0.154	0.671	0.600	0.110	0.191	0.066	0.245
NSBSC11	0.406	0.545	-0.355	0.487	0.522	-0.073	0.186	0.067	0.650	-0.092	0.074	-0.011
NSBSC13	0.443	0.182	0.595*	0.371	0.174	0.537*	0.618	0.400	0.361	0.498	0.022	0.509
NSBSC14	0.729	0.727	0.003	0.680	0.652	0.042	0.770	0.600	0.227	0.077	-0.011	0.066
NSBSC17	0.284	0.227	0.205	0.275	0.217	0.214	0.191	0.200	-0.050	0.163	-0.020	0.146
NSBSC19	0.444	0.182	0.596	0.449	0.217	0.522	0.331	0.400	-0.217	0.408	-0.016	0.399
NSBSC21	0.608	0.500	0.181	0.598	0.652	-0.093	0.515	0.267	0.491	0.143	0.030	0.169
NSBSC22	0.280	0.318	-0.140	0.453	0.565	-0.254	0.248	0.267	-0.077	-0.186	0.022	-0.160
Mean (ALL)	0.523	0.409	0.201*	0.534	0.465	0.146*	0.462	0.387	0.156	0.173*	0.025	0.194
Mean (excl. NA)	0.515	0.443	0.119	0.536	0.506	0.069	0.443	0.383	0.135	0.107	0.021	0.126

H_E, expected heterozygosity; H_O, observed heterozygosity (both H_E and H_O given as proportion of total sample, with MG = 22, MP = 23 and BA = 15); * significant values at $p < 0.05$ level (after Bonferroni correction); NA, loci with putative 'null alleles' (i.e. NSBSC1 & NSBSC13) (van Oosterhout *et al.*, 2004)

Three pairs of loci showed LD: NSBSC7 & NSBSC13 (MG & MP), NSBSC7 & NSBSC17 (MP & BA) and NSBSC8 & NSBSC14 (MP). Genic differentiation between populations was calculated using two separate methods: Pairwise F_{ST} (Weir & Cockerham, 1984) and Fisher's Exact G-test (Raymond & Rousset, 1995) (Table 4.3). Both methods showed significant genic differentiation between MP and BA but not between any other pairs of demes.

Table 4.3 Pairwise tests of genic differentiation between demes

Pairwise F_{ST}	MP	BA	Exact G-test	MP	BA
MG	0.0056	0.0137	MG	0.0682	0.0377
BA	0.0635*		BA	0.0000*	

F_{ST} (Weir & Cockerham, 1984) and Exact G-test calculated from GENEPOP version 4.0.10 (Raymond & Rousset, 1995); * indicates significant values (F_{ST}, $p > 0.05$; Exact G-test, $p < 0.0001$)

To assess whether the sample sizes were sufficient to sample at least a high proportion (if not all) of the available genetic variability, allele discovery curves were constructed (Figure 4.2) for all three demes, based on the random sampling of alleles. Since all three curves quickly reached a plateau, with few new alleles being discovered after ~20 alleles (i.e. 10 individuals) had been sampled, this suggests that, despite sample sizes being below that recommended by Hale *et al.* (2012) for microsatellite diversity analyses, there is confidence that the majority of the available microsatellite variation in these demes has been identified.

Finally, clustering analysis using STRUCTURE to determine what, if any, population structure exists in this dataset was performed. The highest posterior probabilities were calculated for $K = 3$ for both the admixture and non-admixture model (Table 4.4). However, the probabilities from the five runs with the non-admixture model were higher and more consistent with lowest standard deviation between runs than for the admixture model. On face value, these results suggest that the prediction about three genetic populations is correct but on closer examination of the data, this hypothesis can be rejected on the basis that clusters occur within geographic populations (Figure 4.3), not between them, with no true pattern of clustering. This indicates that the 'clusters' modelled in STRUCTURE do not reflect true clusters of genetically similar populations and are probably artefacts of generally

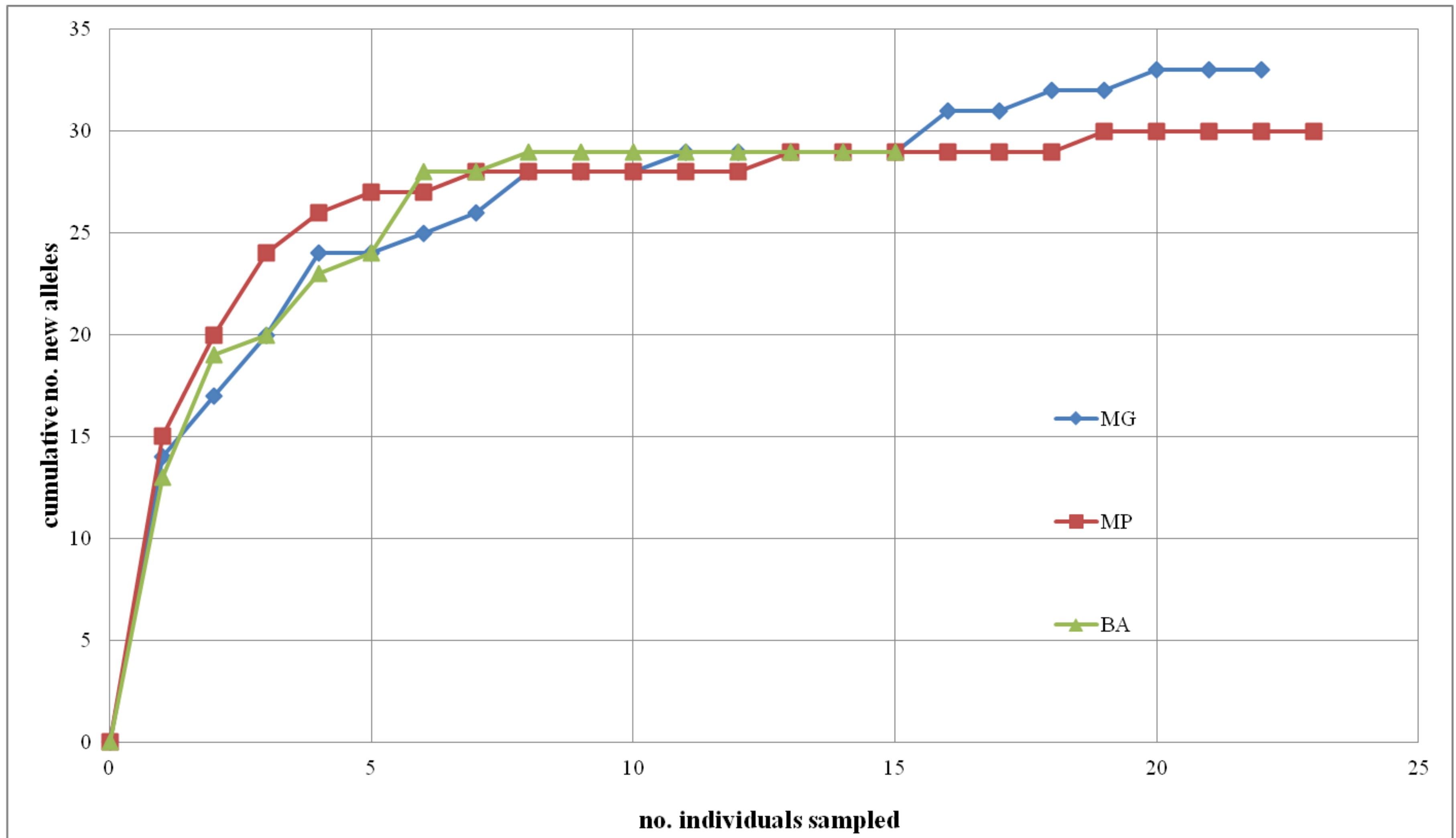


Figure 4.2 Allele discovery curve for 10 polymorphic microsatellite loci in demes MG, MP and BA. Genotypes were sampled at random and new alleles recorded cumulatively.

low genetic diversity across all three geographic populations. However, there was some evidence for geographic clustering within postulated demes MG and MP. Figure 4.4 and Figure 4.5 show clusters within MG and the western sector of MP based on the most differentiated genotypes predicted by STRUCTURE (Figure 4.3) and, although grouping does not result in entirely separate clusters, nevertheless there appear to be clearly defined clusters within each deme. However, the clustering was only observed in the west sector of MP, and was not apparent in the eastern sector.

Table 4.4 Results of population clustering analysis using STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) using both admixture and non-admixture models

Admixture Model			Non-admixture Model		
K	Mean LnP	SD	*K*	Mean LnP	SD
1	-1091.5	0.17	1	-1091.4	0.44
2	-1031.9	8.23	2	-1019.6	0.24
3	-1031.0	4.56	3	-1017.6	1.34
4	-1099.3	10.98	4	-1069.8	5.77
5	-1175.0	33.34	5	-1132.8	11.50

K no. of postulated populations used in STRUCTURE model; LnP Log posterior probability; SD standard deviation based on mean five separate runs per value for *K*. Shaded rows indicate *K* values with highest mean posterior probability for each model

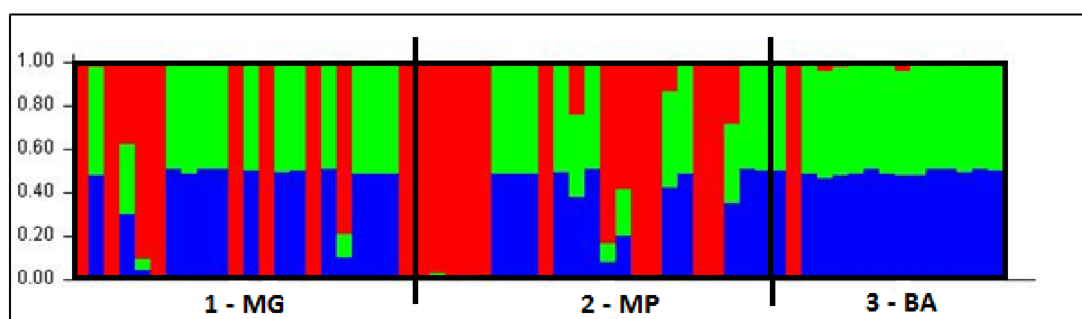


Figure 4.3 Bar plot computed by STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000) for *K* = 3 postulated clusters using non-admixture model with 2,000,000 repetitions (200,00 burn-in) (Log posterior probability of -1015.6). Sections 1, 2 and 3 represent individual NSBs from MG, MP and BA respectively. Coloured segments of each bar represent proportion allocated to each postulated cluster per individual NSB.

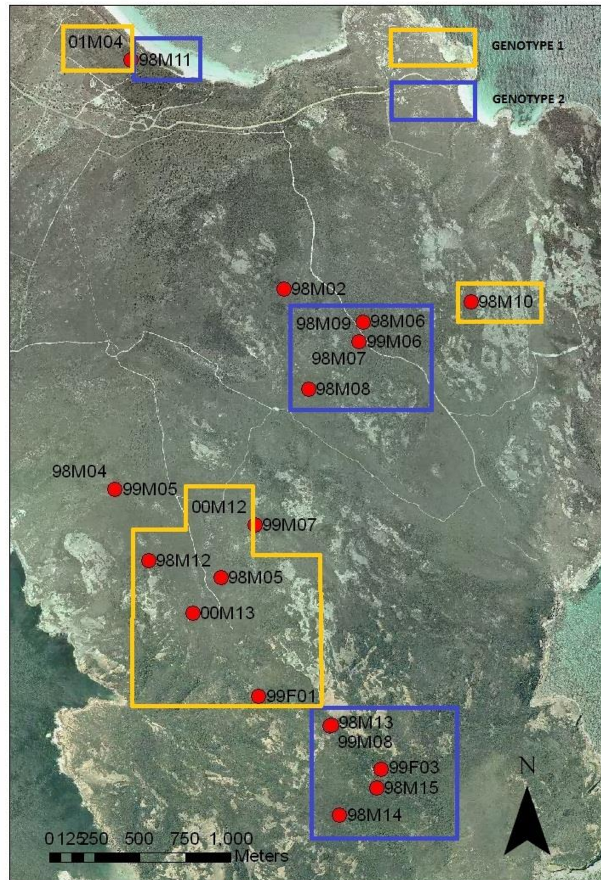


Figure 4.4 Map of MG deme showing geographic location of individual NSB sampled for analysis and which individuals cluster into two of three genotype classes predicted by clustering analysis in STRUCTURE

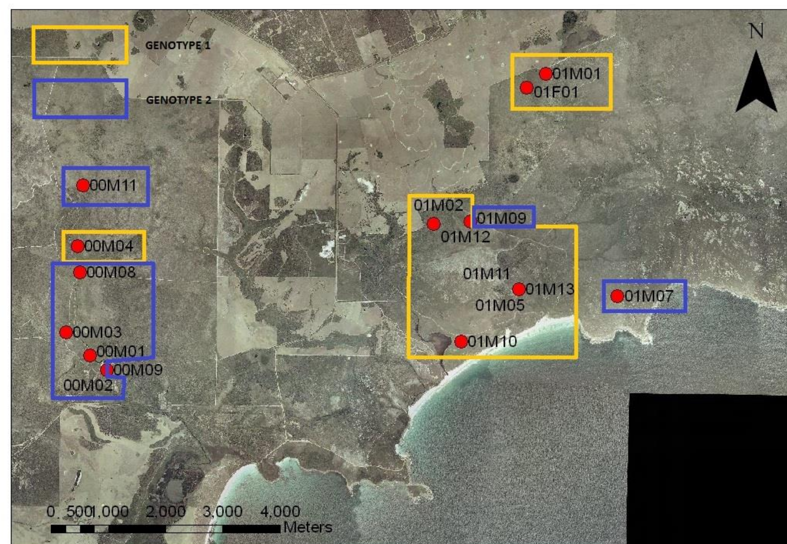
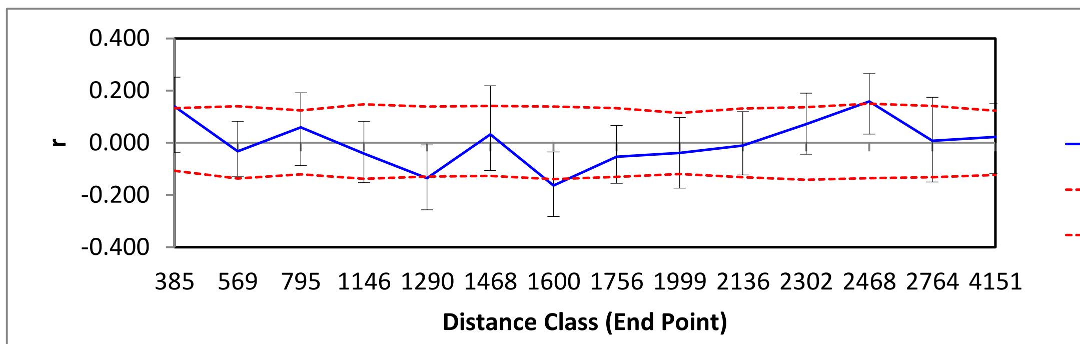


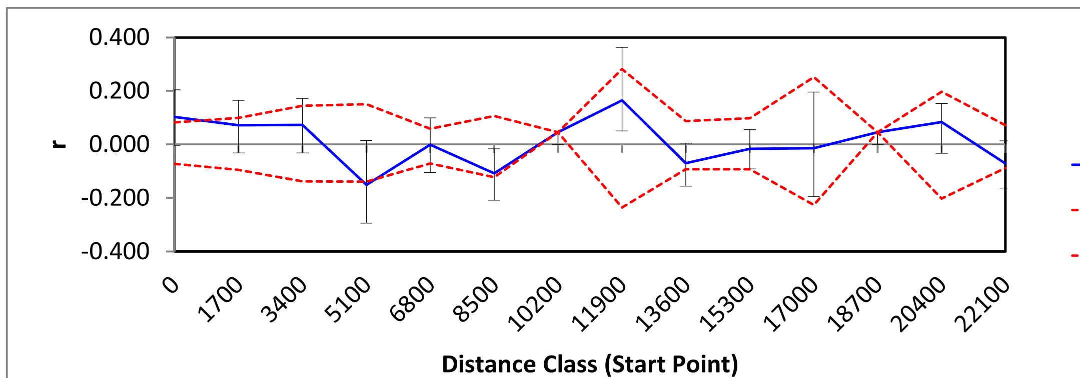
Figure 4.5 Map of MP deme showing geographic location of individual NSB sampled for analysis and which individuals cluster into two of three genotype classes predicted by clustering analysis in STRUCTURE

Spatial autocorrelation analyses (Spatial Structure and Mantel Tests) for all three populations found no strong evidence for a correlation between genetic and geographic distance. If there was a positive correlation we would expect to see than reflected in the shape of the curves in Figures 4.6 and 4.7. Instead, Figure 4.6 shows values for *r* oscillating around 0 rather than decreasing with increasing geographic distance and Figure 4.7 shows either neutral or negative linear regressions.

Mt Gardner



Mt Manypeaks



Bald Island

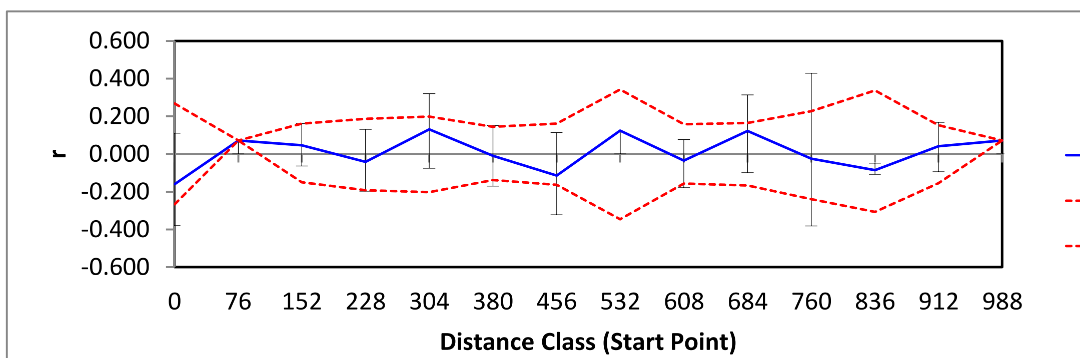
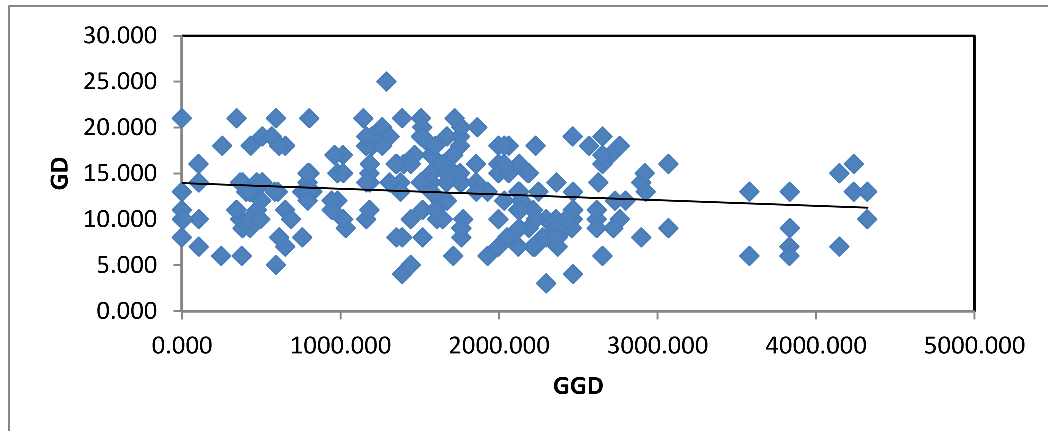
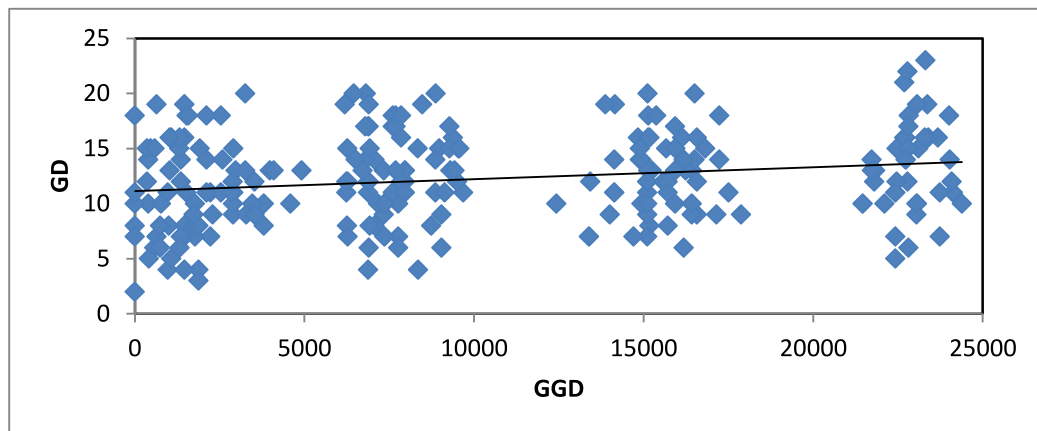


Figure 4.6 Results of Spatial Structure tests for Mt Gardner, Mt Manypeaks and Bald Island using GENALEX version 6.5 (Peakall and Smouse 2006, 2012). *r* is the autocorrelation coefficient and U and L are the upper and lower bounds of the 95% confidence interval.

Mt Gardner



Mt Manypeaks



Bald Island

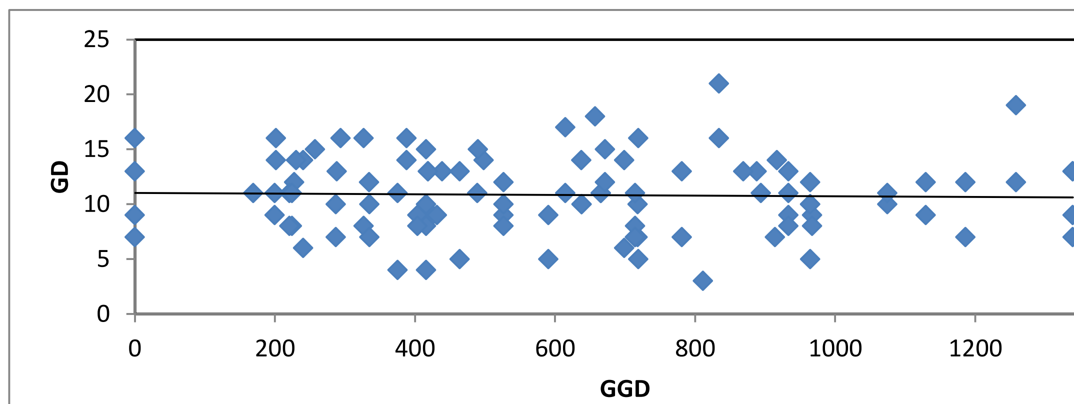


Figure 4.7 Results of Mantel tests for geographic distance (GGD) vs. genetic distance (GD) for Mt Gardner, Mt Manypeaks and Bald Island using GENALEX version 6.5 (Peakall and Smouse 2006, 2012).

Population assignment with GENALEX also failed to consistently assign individuals to the 'correct' population, with only 31 out of 60 individuals assigned (52%) to their 'own' population.

The results of effective founder size modelling for BA suggested that there were 9.2 reproductive founders out of 11 individuals released. However, confidence intervals for 95% (4.9 – 14.2) and 90% (6.4 – 12.5) varied widely compared to other studies (Kalinowski *et al.*, 2010; Alda *et al.*, 2013) with the upper confidence interval rising above the known number of founders, suggesting this result may not be very accurate.

Modelling with BOTTLENECK suggested a bottleneck for the MP (for IAM and SMM models and a mode-shift in the L-shape distribution) (Table 4.5) and MG (IAM only) populations but both the MG and BA demes did not show a mode shift, which was somewhat surprising given what is known about the history of these populations, especially the latter.

Table 4.5 Results of analysis for detection of bottlenecks using BOTTLENECK version 1.2.02

	Wilcoxon Test			
Subpopulation	IAM	SMM	TPM	L-shape distribution
Mt Gardner	0.01367*	0.16016	1.00000	normal
Mt Manypeaks	0.00684*	0.01855*	0.27539	mode-shift
Bald Island	0.19336	0.55664	0.92188	normal

BOTTLENECK mutational models: IAM, infinite alleles model; SMM, stepwise mutation model; and TPM, Two-phase Model; * indicates statistically significant values

Ne-ESTIMATOR predicted that the N_e for MG for the approximate time period that samples were obtained for this deme (1998-2001) was 46.8 (LD method) or 68.0 (Molecular Coancestry method). The Heterozygote Excess method result was infinite and therefore not useful. The values for the two other methods appear potentially valid but the 95% confidence intervals for these methods fluctuate between 9.3 and infinity, which somewhat reduces their validity.

4.4 Discussion

4.4.1 Intra- and inter-population genetic diversity in MG, MP and BA

Population bottlenecks are understood to cause reductions in genetic diversity, which in turn have been shown to positively correlate with extinction risk (Frankham, 2005; Evans & Sheldon, 2008) due to the associated loss of fitness (e.g. O'Brien *et al.*, 1987; Roelke *et al.*, 1993). However, there is the theory that such bottlenecks can

purge recessive deleterious alleles by exposing them to natural selection (Keller & Waller, 2002; Leberg & Firmin, 2008), which can in turn reduce inbreeding depression. Although the NSB may possibly satisfy some of the requirements for purging (e.g. gradual inbreeding and isolated populations (Keller & Waller, 2002) there is minimal evidence for this in any wild animal populations. Furthermore, Leberg & Firmin (2008) presented a review of purging in captive breeding and restoration programmes, combined with their own data, and found that the effects of bottlenecks are highly unpredictable and putative benefits of purging may be outweighed by the negative influence of bottlenecks on fitness (e.g. fixation of deleterious alleles leading to inbreeding depression).

The NSB is understood to have undergone one protracted bottleneck during its apparent 'extinction' between c.1900 and 1961, which has been theoretically compounded by the translocation of small founder groups to new locations (serial population bottlenecks). Ideally, the translocation of NSBs to e.g. Mt Manypeaks and Bald Island should have used many more founders than the 32 and 11 respectively but, as with most programs, lack of resources was a crucial limiting factor, which was compounded by the NSB being a highly elusive and cryptic species. This study sought to answer questions relating to the downstream genetic variability in the three demes, (between which there is limited or no gene flow due to fragmentation or geographical barriers), i.e. what genetic diversity still remains across and within all demes, and to what extent has genetic differentiation occurred between demes.

Ten polymorphic microsatellite loci, developed specifically for the NSB (Chapter 3), were used to answer these questions. Small founder size (instantly reducing the amount of genetic variation available) combined with the loss of genetic variability from drift and inbreeding processes (Keller & Waller, 2002) will increase the likelihood of a reduction in genetic diversity in translocated populations (Mock *et al.*, 2004). The results support this, as diversity across all 10 loci was found to be generally low, with the highest figure for MAL being just 3.3 for MG with a MAR (which adjusts for sample size) of just 3.15. This matched the earlier prediction that the parental deme (MG) should have more variability than the two translocated demes (MP & BA), with BA being the least diverse (MAL/MAR = 2.9). Furthermore, in BA two di-allelic loci appear to be heading towards fixation (Figure 4.6) and at least eight loci have rare alleles in one or more deme that may be lost over time through drift. However, the low allelic diversity overall suggests generally low

Table 4.6 Allelic diversity (mean alleles/locus) in a range of threatened, near threatened and non-threatened bird species

Species	Scientific Name	Bottleneck Size	Loci	Allelic Diversity (mean alleles/locus)	Reference
white-fronted chat	*Epthianura albifrons*	na	18	17.10	King *et al.*, 2012
greater prairie-chicken*	*Tympanuchus cupido*	na	6	8.5-10.5‖	Johnson *et al*., 2003
hazel grouse	*Bonasa bonasia*	na	8	7.63	Rutkowski *et al.*, 2012
Krüper's nuthatch†	*Sitta krueperi*	na	8	5.75	Albayrak *et al.*, 2012
Rarotonga monarch*	*Pomarea dimidiata*	29	7	4.00	Chan *et al.,* 2011
Réunion cuckoo-shrike*	*Coracina newtoni*	ongoing	17	3.82	Salmona *et al.*, 2012
Asian crested ibis*	*Nipponia nippon*	7‡	11	3.30	He *et al.,* 2006
noisy scrub-bird*	*Atrichornis clamosus*	c.45 males	10	3.30	This study
saddleback†§	*Philesturnus carunculatus carunculatus*	1000	7	3.17	Taylor & Jamieson, 2008
Laysan finch*	*Telespiza cantans*	100	10	3.11	Tarr *et al.,* 1998

* listed as threatened (Vulnerable/Endangered/Critically Endangered) by IUCN (2012) (www.iucnredlist.org); † listed as Near-threatened by IUCN (2012); ‡ Chinese population only; § South Island subspecies only; ‖ reflects variation between state-wide populations in Kansas, Minnesota, Missouri, Nebraska and Wisconsin only

genetic diversity across all demes, and this concurs with studies in other species known to have undergone major bottlenecks (Table 4.6). The low overall diversity of the NSB is comparable with other bird species like Réunion cuckoo-shrike (*Coracina newtoni*), Asian crested ibis (*Nipponia nippon*) and Rarotonga monarch (*Pomarea dimidiata*), all of which either are or previously were listed by IUCN as Critically Endangered and in imminent danger of extinction (IUCN, 2012).

The significant deviations from HWE and inbreeding coefficient (F_{IS}) values ($p < 0.05$) predicted for MG and MP, indicate that these populations are inbred. Removal of two loci that were predicted as having putative 'null alleles' reduced F_{IS} values below the $p < 0.05$ significant threshold for MG and MP (and the overall value for all populations). As these 'null alleles' were not predicted for BA, this suggests that the heterozygote deficiency observed for MG and MP is a true reflection of the inbreeding in these populations. Somewhat surprisingly, F_{IS} is not significant for BA and, although the sample size for this deme was smaller than the other demes (n = 15), Table 4.2 shows that four out of 10 loci were actually in heterozygote excess. Heterozygote excess is understood to occur in small populations, under the influence of binomial-sampling error (Pudovkin *et al.*, 1996; Luikart & Cornuet, 1999; Balloux, 2004; Allendorf & Luikart, 2007). For example, when small founder groups are sampled randomly for translocation, differing genotypes in males and females that subsequently go on to reproduce willincrease the number of heterozygotes in subsequent generations. In the case of BA, a heterozygote excess at 40% of loci tested is suggestive of a recent population bottleneck (Cornuet & Luikart, 1996), confirming what is already known about this population. Heterozygote excess has been maintained in this population since foundation in 1992/1993 and may be explained by the strong population growth experienced in this population. Values for F_{ST} for all loci were generally low (-0.020 to 0.074) with an average of 0.025 indicating that the values for F_{IS} relate to within-population inbreeding rather than population subdivision.

Pairwise estimates of population subdivision (F_{ST} and Exact-G Test) found significant differentiation between MP and BA, the two demes derived from separate translocations. This is not unexpected given that random-sampling error combined with difference in allele frequency distribution, particularly with small founder groups, can lead to derived populations with differing genotypes (Fuerst & Maruyama, 1986; Taylor & Jamieson, 2008). How biological significant this result is

unclear but the significant differentiation could potentially be important from a conservation management viewpoint, as this is something that is not desirable in the establishment of new populations.

Significant LD was found in three pairs of loci inconsistently across all three demes (MG = 1; MP = 3; BA = 1). LD can often be associated with severe population bottlenecks and may persist in the long term (Wang *et al.*, 1998) so a degree of linkage would be expected in all three demes.

The dataset was divided into three discrete demes (MG, MP & BA), believed to reflect the three geographic and genetic populations, assuming minimal gene flow between MG and MP and no gene flow between either MG or MP and BA, due to Bald Island's geographic isolation. However, the cluster analysis performed using STRUCTURE did not support this hypothesis, despite predicting three genetic clusters, as clustering was predicted within populations but not between them. Therefore, the inferred clusters are most probably a computational artefact resulting from a combination of observed low levels of genetic variability compounded by low sample size, which may not provide high enough resolving power to elucidate potential population structure. Therefore, in the absence of any strong evidence fo genetic clustering within and between postulated demes, these subdivision units appear to be functionally arbitrary and should only be used as geographic management units rather than genetic management units. However, the clustering that was observed in the MG and MP demes does highlight the importance of sampling for translocations over a wide geographic area, in order to maximise the genetic variability in the founder group.

The lack of evidence of geographic population structure was corroborated with results from spatial autocorrelation analyses, which showed no evidence for any kind of relationship between genetic and geographic distance, either positive or negative. Furthermore, population assignment using GENALEX version 6.5 (Peakall and Smouse 2006, 2012) also supports the observed results in STRUCTURE in that there was minimal evidence for any geographic population structure, with just over half of all individuals sampled assigned to the same population they actually came from.

Microsatellite data was used to undertake population genetics modelling for founder size (BA), N_e (MG) and bottlenecks (all demes). Estimates for founder size and N_e were within expectations, with approximately nine individuals (from 11 released) estimated to have established BA and N_e for MG (between 1998-2001 when birds

were sampled from this deme, although 19 of 22 individuals were sampled in 1998 and 1999) was estimated to be 4.9 to 14.2. The estimate of 9 founders from 11 released individuals on BA would help to explain why allelic richness is not much lower than that observed for MG and MP (2.90 vs. 3.15 & 3.00 respectively). The estimate for N_e for MG is relatively low, with a census size for this period of 131-166 territorial males (Figure 1.2), but biasing due to uneven mating success between sexes (e.g. in a polygamous or polygynous mating system, which we hypothesise for the NSB (1.5.1)) can skew estimates of N_e (Wright, 1938; Nunney, 1993). This has been shown experimentally in a polygynous bird species (Gunnison's sage-grouse (*Centrocercus minimus*)), where a population had an estimated N_e of 42, representing just 19% of the estimated total population (Stiver *et al.*, 2008). This is under the threshold of 50, below which inbreeding depression would be predicted to become important (Franklin, 1980; Soulé, 1980). Given the hypothesised polygynous mating system of the NSB, it might be expected that estimates of N_e will be significantly lower than the population estimate. Danks *et al.* (1996) suggest a rule-of-thumb of 2.5 times the estimate of number of territorial males, which would make the average total population 371 over the temporal range of the sample from MG. 46-68 individuals would represent just 12-18% of the total population, although there are two considerations that require caution with this estimate: a) the estimated 95% confidence interval fluctuates widely, from 9.3 to infinity and b) there is no way to verify the estimate of total population size, particularly for this highly elusive species. Therefore, no firm conclusions can be drawn from this. However, this is interesting considering the estimate for N_e is so low, particularly in light of the results presented by Stiver *et al.* (2008) and the hypothesised mating system of the NSB. Additionally, given that estimates of F_{IS} suggest that the MG population has perceptible inbreeding, the implications of low N_e in this deme are potentially important since this may impact on the ability of this population to recover. Furthermore, if inbreeding depression is an issue for this population, it would be expected that there may be an increase in disorders such as 'rubber-bill' syndrome.

The modelling of putative bottlenecks was less successful, as while three models (IAM/SMM/mode-shift) predicting that the MP population had had a recent bottleneck, only one model predicted a bottleneck for MG (IAM) and no bottleneck was predicted for the BA, which we know was established with just 11 individuals. While the MP population may have experienced a recent bottleneck from the 2005

wildfire, the temporal range of the MP sample set is mostly prior to this event, with just 6 individuals sampled post-fire and those from the Mermaid subpopulation which was unaffected directly by the fire. The recommended minimum number of loci for use in BOTTLENECK modelling is 20 and while Spencer *et al.* (2000) found that just eight loci was sufficient to model a recent bottleneck, these loci were highly polymorphic, which may not be the case for a species such as the NSB. Therefore, it may be that the 10 loci presented in this study are insufficient to provide the resolution required by the models used by BOTTLENECK.

Although the sample sizes were slightly below the ideal threshold for microsatellite diversity analyses set by Hale *et al.* (2012), allele discovery curves (based on random sampling of alleles) were constructed for all demes, which suggest that a high proportion of available genetic variation was sampled even from these relatively small cohorts.

4.4.2 Implications for conservation and management

The genetic diversity at the 10 microsatellite loci used in this study provides a window on the overall genetic variability remaining in the NSB as a species. The maximum allelic diversity of 3.3/3.13 (MAL/MAR) for MG puts the NSB in the same league as some of the rarest birds in the world, some of which have undergone even more extreme bottlenecks. Furthermore, inbreeding coefficient values for MG and MP indicate both of these populations are inbred. However, in spite of this, it is somewhat surprising that MP and BA have appeared to thrive with strong population growth observed for both locations. In contrast, MG, which has exhibited a decrease in population size, the low diversity is potentially compounded by a low estimate of effective population size, which will increase the probability of inbreeding and may increase the risk of inbreeding depression. This may help to explain why the MG deme has not performed particularly well in recent years. BA has lower values for heterozygote deficiency, in part due to the heterozygote excess in 40% of loci tested, but this is likely to be caused by binomial-sampling error in the major founder event when the deme was founded with just 11 birds (including 3 females), although it is expected that maybe only 9 of these birds actually successfully reproduced. This is underlined by this deme having the lowest allelic diversity of 2.9 (MAL & MAR). LD was noted in all demes but mainly in the MP population where 3 pairs of loci showed significant LD. Finally, if statistically significant genic differentiation

between MP and BA is also biological significant, this could potentially be an issue for in the future management of these populations.

Although variation between MG and the two translocated populations is statistically significant ($p < 0.01$), the real-life differences are relatively low with minimal loss apparent through translocations (i.e. SPBs). This suggests that the founder groups used in these translocations, although very small, were sufficient to capture a large proportion of the available microsatellite diversity in the donor population (MG).

Given the variable temporal nature of the sample sets for these demes, much of what can be seen in this analysis may not be contemporarily accurate, except perhaps for BA (sampled from 2009-2011). However, previous knowledge of the population dynamics of these demes since tissue samples were taken allows extrapolation to the present-day. The Lakes meta-population in the MG deme was effectively extirpated in 2000 by a wildfire and there is limited evidence of re-colonisation in the interim. The Lakes is a significant population for two reasons: a) it represents a sink for population immigration into this population which is thought to be dependent on productivity in the Mt Gardner source meta-population; b) it improves connectivity between the MG and MP demes as there is evidence NSBs will pass through the corridor between the Lakes and the Angove Water Reserve (Danks *et al.*, 1996). Therefore, the long-term absence of a NSB in the Lakes means a restriction on the movement of genes between MG and MP demes and may also reflect reduced productivity on Mt Gardner. More evidence for the latter comes from the decline of territorial males on Mt Gardner from 170 in 2002 to 102 in 2014. In light of the genetic evidence for inbreeding and potential inbreeding depression in this deme, this decline may be a significant cause for concern.

While the MP deme has shown stronger population growth, the impact of the extensive wildfire on Mt Manypeaks in 2005 remains unknown. Since the fire, the number of active territories has increased from 60 in 2006 to 146 in 2011, which suggests this deme remains healthy. However, it is unknown whether the 2005 wildfire represents a significant bottleneck, further impacting this deme, which is already understood to be inbred with low genetic diversity. The BA population, by contrast, appears to have been more successful. Since inception in 1992-1993 the population has grown strongly and, possibly due to a combination of binomial-sampling error in the first instance (as discussed above) and rapid population growth, this population has avoided a significant deviation from HWE.

Furthermore, as the structural relationships between all three demes were shown to be quite weak, there did appear to be some clustering within demes, which, although not continuous enough to be considered a cryptic population structure, did underline the need to assemble founder groups for translocation from as wide a geographic area as possible.

So, while this study has answered questions relating to the inter- and intra-population genetic diversity and inbreeding of the NSB, it raises more questions about the conservation implications. In spite of their low genetic diversity, the MP and BA populations continue to show healthy growth, while the (slightly) more diverse but more inbred MG deme stagnates. Is the growth in MP and BA sustainable and how has BA managed to retain genetic diversity in the face of a major founder event and the inferred effect of genetic drift? Can the inbreeding and genic differentiation observed be alleviated by assisted migration between populations and where should translocation efforts be directed in future? Certainly, maintaining these three populations should remain a high priority, as should the recovery of the MG deme to the point that there is once again gene flow between MG and MP. Artificial gene flow between the mainland populations and BA should also be considered to consolidate the gene pool of this important population.

Furthermore, it should also be considered that there may other factors that could be more important in influencing extinction risk in this species other than genetic diversity. Little is known of the demography of the populations of NSB and in some cases demographic factors may be more important than genetic diversity and inbreding in evaluating extinction risk (Lande, 1988). Threats listed as significant for the NSB include predation by feral cats and climate change but there may be other threats that could be influential in e.g. the decline of the MG population. However, while it is difficult to speculate on the nature of these factors, it is important to consider that genetic diversity may not provide the whole story.

Nevertheless, to truly understand the ecological and conservation significance of such apparently low genetic diversity, data from the analysis of genes representing quantitative traits (or adaptive variation), which have been shown to be of more utility in population studies than neutral markers alone (Hedrick, 2001), should be considered.

Chapter 5 – Diversity of the Major Histocompatibility Complex Class II B gene in the noisy scrub-bird

The Major Histocompatibility Complex (MHC) is a gene complex considered to be of high immunological significance potentially influencing a range of other fitness traits included fecundity and mate choice. Therefore, this gene group represents an important group of markers that can be used to measure levels of adaptive variation. Using degenerative primers and DNA amplification in combination with blue-white *E. coli* cloning selection techniques, sequences were generated from the [exon 2 - intron 2 - exon 3] region of MHC Class II B gene of the NSB. A total of 16 independent clones were sequenced from 10 individual animals from each of the three main subpopulations (30 total). A high level of sequence variation is observed for this gene in this species. Furthermore, strong evidence is presented supporting balancing selection, suggesting that the observed diversity is driven through evolutionary processes. This represents the first research into adaptive genes in the NSB and the high levels of variation in the MHC Class II B observed may be of major significance to the future conservation of this and other threatened species.

5.1 Introduction

In conservation genetics, levels of neutral variation (e.g. microsatellites) may not always correlate with adaptive variation, (i.e. genes and loci that confer a fitness advantage and will therefore be under selection) (Hedrick, 2001). While many genetic diversity studies have used microsatellites (e.g. in birds, (see Table 1.1)), at a landscape scale, neutral loci may be more valuable for examining processes such as gene flow and population/subpopulation structure (Holderegger *et al.*, 2006), but are not ideal for predicting evolutionary potential (Hedrick, 2001; Holderegger *et al.*, 2006), the loss of which may be a key factor for increased extinction risk (Frankham, 2005). Therefore, to fully assess the conservation genetics of the noisy scrub-bird, adaptive loci in the species' genome should also be examined. However, many quantitative traits are dependent on covariance within a large number of minor genes or quantitative trait loci (QTLs). These QTLs may show less differentiation than

neutral markers and therefore may not be ideal subjects for assessing adaptive variation in a genome (Latta, 1998). In contrast, genetic diversity in QTLs may actually be enhanced by bottlenecks as, after an initial decrease, additive variance increases through the conversion of epistatic variance (Goodnight, 1988; Cheverud & Routman, 1996). Therefore, while single neutral loci may experience a loss of genetic diversity after a bottleneck, diversity in QTLs may be increased by this mechanism. Given the unpredictability of the behaviour of QTLs in response to bottlenecks, it is important to be aware of their potential influence on other adaptive gene groups.

The Major Histocompatibility Complex (MHC; known as the Human Leucocyte Antigen (HLA) in humans) represents a popular gene target choice in conservation genetics studies (Ujvari & Belov, 2011) and has already been used to investigate genetic diversity in a wide range of bird species e.g. Miller & Lambert, 2004a; Bollmer *et al.*, 2005; Miller *et al.*, 2011 etc (Table 1.1). The MHC's popularity as an adaptive gene used in genetic diversity studies stems from the following factors: a) the MHC contains some of the most polymorphic gene groups in the vertebrate genome (Penn, 2002a); b) most genes are codominantly expressed, so that both alleles at a given locus will be expressed, and this, combined with the highly polymorphic nature of MHC classical genes, will result in the expression of a large number of different MHC molecules in a population (Janeway *et al.*, 2001; Coico *et al.*, 2003); c) it plays a crucial role in regulating immune function through the development of T lymphocytes (or T-cells) and the immunological response of T-cells (Coico *et al.*, 2003) in self/non-self-recognition (Penn, 2002a) and represents a fitness-related quantitative trait that may exhibit selection. Aside from its important role in pathogen resistance (Penn *et al.*, 2002; Owen *et al.*, 2008; Oppelt *et al.*, 2010; Biedrzycka *et al.*, 2011), diversity in the MHC has also been shown to influence a range of other fitness-related traits (Ujvari & Belov, 2011), e.g. maternal-foetal interactions (Thomas *et al.*¸ 1985); lifetime reproductive success (Kalbe *et al.*, 2009). However, there is evidence that non-MHC genes (i.e. QTLs) have a larger cumulative contribution than MHC genes themselves (Jepson *et al.*, 1997). Therefore, it must be remembered that MHC diversity is merely an indicator of adaptive diversity in this gene group and any conclusions must account for this. Furthermore, there are other limitations to using MHC sequences to assess genetic diversity, such as the possible presence of pseudogenes, which may not be easily

differentiated from functional sequences. In addition, assignment of alleles to loci is also difficult and has only been achieved in a small number of bird species. Therefore, the 'best guess' for a minimum number of loci is most easily obtained from the number of alleles obtained from a single individual (e.g. Bollmer *et al.*, 2010).

Since the MHC has a key role in regulating the immune system, selection of variants at this gene group is thought to be driven by the advantage to the organism through the resulting resistance conferred by an allele for a specific pathogen. This provides a selective advantage to that individual and hence drives the relatively polymorphic content observed in this gene group (Jeffrey & Bangham, 2000). The most likely explanation is either through balancing selection (Jeffrey & Bangham, 2000), in which a heterozygote has a distinct advantage (codominance selection) (Hughes & Nei, 1989; McClelland *et al.*, 2003) or alternatively the rare-allele advantage (Sutton *et al.*, 2011), where higher diversity between alleles results in greater fitness. Balancing selection has been thought to be able to maintain polymorphisms, for example, in otherwise monomorphic species such as the island fox (*Urocyon littoralis*) (Aguilar *et al.*, 2004).

However, other processes are understood to also be important in driving MHC diversity. Mate choice (or sexual selection) has also been shown to be important (Penn & Potts, 1999; Penn 2002b) and a review by Kamiya *et al.* (2014) found MHC dissimilarity and diversity was a statistically significant influence in mate choice. Eizaguirre *et al.* (2009) showed experimentally the role of olfactory cues in MHC-mediated mate choice in three-spined sticklebacks (*Gasterosteus aculeatus*), although the work of McCairns *et al.* (2011) suggested that it may be secondary to other processes. The same olfactory cues have also been associated with HLA and mate choice in humans (e.g. Thornhill *et al.*, 2003). There is also evidence that MHC-mediated mate choice can occur in birds, e.g. in the blue petrel (*Halobaena caerulea*), a long-lived, socially monogamous species with well-developed olfaction, but only with respect to the Class II MHC (Strandh *et al.*, 2012). Another process that has been linked to MHC polymorphic diversity is gene conversion, which acts to increase allelic diversity (Ohta, 1991), often at a faster rate than the accumulation of point-mutations (Parham & Ohta 1996). Gene conversion has been shown experimentally to generate increased variability in a genetically depauperate bird species, Berthelot's pipit (*Anthus berthelotii*) (Spurgin *et al.*, 2011). Gene conversion

has also been suggested as a key driver of the extraordinary MHC diversity observed in the common yellowthroat (*Geothlypis trichas*) (Bollmer *et al.*, 2010).

The importance of diversity in genes such as the classical MHC genes is apparent in all species but has particular importance in small, bottlenecked or potentially inbred populations (Oliver & Piertney, 2012). Species that have undergone bottlenecks or have been reduced to small populations commonly show relatively low MHC diversity (O'Brien *et al.*, 1985; Hoelzel *et al.*, 1999; Hedrick *et al.*, 2000; Miller & Lambert, 2004a; Smith *et al.*, 2010a etc) and in these cases the MHC diversity correlates with a concomitant lower neutral variation (Zhang et al., 2006; Mason *et al.*, 2011). However, a few studies of the MHC in isolated and highly threatened species have shown considerably higher than expected levels of diversity, most likely because of pathogen-driven balancing selection (Aguilar *et al.*, 2004; Du et al., 2010). Nevertheless, while selection may alleviate some of the loss of variation during a bottleneck, modelling has shown that selection cannot be relied upon to maintain or increase variability in a genetically-depleted population (Ejsmond & Radwan, 2011).

In Chapter 4 it was shown that, in 10 neutral (microsatellite) loci, the NSB has relatively low diversity (maximum allelic richness of 3.19), which is comparable with similar findings obtained from some of the rarest bird species on Earth. However, as previously discussed, it cannot be confidently be inferred that low neutral variation correlates directly with low adaptive variation. To better investigate levels of adaptive variation, which is potentially more important given its relationship with evolutionary potential (and therefore extinction risk), diversity in the NSB at the MHC Class II B was examined. Based on the low microsatellite diversity observed for other bottlenecked bird species at both microsatellite and MHC loci (e.g. black robin (Miller & Lambert, 2004a); asian crested ibis (He *et al.*, 2006; Zhang *et al.*, 2006); Galápagos hawk (Bollmer *et al.*, 2011); Seychelles warbler (Richardson *et al.*, 2000; Richardson & Westerdahl, 2003); little spotted kiwi (Miller *et al.*, 2011)), it is therefore predicted that there will be comparably lower levels of MHC diversity in the NSB. Furthermore, species that display a higher than expected MHC diversity (e.g. island fox) also have been observed to have a history of recurrent epidemics of disease (Aguilar *et al.*, 2004), which is thought to drive their MHC diversity through balancing selection. Pathological studies of the NSB individuals used in recent translocations showed no observable evidence of

significant disease, (Edmonds, 2007 unpublished). Therefore, as there are no clearly identifiable pathogens that might drive contemporary MHC diversity in the NSB a lower diversity is also expected. Inter-population comparisons between the three postulated demes are also hypothesised to show relatively higher diversity in the parent population at Two Peoples Bay (MG), when compared to the two translocated demes on Mt Manypeaks and surrounds (MP) and Bald Island (BA), which were established with relatively small founder groups.

5.2 Methodology

5.2.1 Amplification and sequencing of MHC Class II B loci

Genomic DNA samples were extracted from NSB blood and feather tissue samples (as per 2.3.1 & 2.3.2). Amplification of MHC Class II B exons 2 and 3 was attempted using a range of primers sets, including degenerative primers developed by D. Giustiniano (*pers. comm.*) (Table 5.1). Exon 2 was successfully amplified by PCR (2.4.1) using the primer sets PEN1/PEN2 and P1/P2 and Exon 3 was successfully amplified using AlEx3F/R and AlEx3F/Exon3R. However, the BRMHC05/BRMHCex2int1 primer set was not successful in amplifying Intron 1. Amplification of the region Exon 2 – Intron 2 – Exon 3 was attempted using P1/PEN1 as forward primers and AlEx3R/Exon3R as reverse primers and the combination of P1/AlEx3R was found to successfully amplify this region, although it was necessary to increase the number of PCR cycles to obtain a sufficient product for sequencing. Gel electrophoresis showed that the use of the high number of PCR cycles resulted in a very strong primer dimer band being present. However, this primer-dimer band was eliminated from subsequent ligation and transformation through the use of gel extraction (2.3.3).

Burri *et al*. (2014) provided recommendations for maximising the success of amplification of the avian MHC Class II B. Whilst the experiments in this present study were undertaken prior to this publication, many of the recommendations were serendipitously implemented in the process of optimising the PCR techniques. Regarding primer choice, Burri *et al*. (2014) recommend checking primers against the latest sequence data and consider using specific primers. Since various combinations of primer pairs were tested, a combination could be selected that provided sequence data that gave a strong BLAST match with MHC Class II B in

Table 5.1 List of primers tested for amplification of the MHC Class II B region of the Noisy Scrub-bird genome

Primer	Forward/Reverse	Sequence	Region	Species	Reference
P1	Forward	AAG GBC SAG TGY TAC TWY ABB AAC GG	Exon 2	*Chinemys reevesii*	En *et al.* (2008)
P2	Reverse	TAG TTG TGS CKG CAG WSG TGT C	Exon 2	*Chinemys reevesii*	D. Giustiniano (2010)*
PEN1	Forward	AAC GGC ACC SAG CGG GYG AGG T	Exon 2	*Tyto alba*	D. Giustiniano (2010)†
PEN2	Reverse	CYC GTA GTT GTG YYG GCA G	Exon 2	*Tyto alba*	D. Giustiniano (2010)†
Exon 3	Reverse	TCC AGC ATC ACC YGG AWC TG	Exon 3	generic	D. Giustiniano (2010)
AlEx3F	Forward	TGC TMC GTG MYG GRY TTC TAC CC	Exon 3	generic	Alcaide *et al.* (2007)
AlEx3R	Reverse	CAC CAG CAS CTG GTA SGT CCA GTC	Exon 3	generic	Alcaide *et al.* (2007)
BRMHC05	Forward	CGT RCT GGT GGC ACT GGT GGY GCT	Intron 1	*Petroica traversi*	Miller & Lambert (2004a)
BRMHCex2int1	Reverse	GCC CCA CAT CGC TGT CGW ACC T	Intron 1	*Petroica traversi*	Miller & Lambert (2004a)

* En *et al.* (2008); † Modified from Burri *et al.* (2008)

passerine bird species in the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore), providing confidence that the correct region was being amplified. Another recommendation was the use of enhancement additives in the PCR master-mix to facilitate robust amplification of GC-rich regions together with the use of a 'hot-start' Taq polymerase. In PCR master-mixes typically either Bovine Serum Albumin (BSA) (which has been shown to enhance PCR efficiency in GC-rich templates (Farell & Alexandre, 2012)) was used or the Taq polymerase contained additives, both assisting the amplification of GC-rich templates. The use of a 'hot-start' 'enhanced-fidelity' Taq polymerase (MyFi (Bioline)) was used. Burri *et al*. (2014) also advocate the use of high annealing temperatures (up to 68ºC) combined with longer denaturation times (10 mins initial/40 secs cycling). Whilst slightly shorter denaturation (6 mins/20 secs) and lower annealing temperatures (59/57ºC) were used in this study, these are still relatively long/high and it was empirically found they were more than adequate for successful PCR amplification. Furthermore, gel extraction of candidate bands (also suggested by Burri *et al*., 2014) was also performed as this eliminates any interference from strong primer dimers.

Since the amplification of multiple alleles and loci was expected, cloning was required. Gel extracted PCR products were ligated into pGEM (2.7.1) and then subsequently used to transform competent *Escherichia coli* cells (2.7.2). Positive clones (white colonies) were picked and cultured prior to plasmid extraction (2.7.3). The extracted plasmids were screened for inserts using PCR and using the M13F/R primer set. Plasmids containing the correct size insert were submitted for sequencingand sequenced in the forward and reverse directions using the M13-pUC universal primer sets used by Macrogen Inc. (Korea). Sequences were viewed in AB1 format and imported into CodonCode Aligner software version 4.0.4 (Codon Code Corporation). Prior to any analysis poor quality end sequences were clipped and vector "pGem-TEasy" sequences removed.

Genomic DNA samples from 10 individuals from each deme (MG, MP & BA, 30 samples in total) (Appendix i) were PCR amplified and 16 clones were randomly selected for each individual for sequencing, generating a total of 480 individual sequences. This number of clones was selected based upon other similar studies and also supported by the recommendations of Burri *et al*. (2014). The large number of

sequences generated in this project was presumed to be sufficient for the successful capture of all MHC Class II B diversity within the NSB genome.
Some potential pitfalls of sequencing a range of unknown sequences are the occurrence of PCR-induced artefacts, sequencing errors and presence of chimeric clones. Minimising the impact of PCR errors was sought by using multiple amplifications per individual and by comparing the sequences resulting from amplification by MyTaq and MyFi (high-fidelity) Taq polymerase. The latter should have a lower native error rate and from this deduce the rate of error from using MyTaq. Chimera formation during PCR was reduced by using longer extension times, thus preventing the presence of short products which could reprime. In addition, sequencing errors and chimeras were also screened, post PCR, visually in the alignments of the cloned sequences using CodonCode Aligner.

5.2.2 Analysis of MHC sequence data

Sequences were analysed using FGENESH (Softberry) to predict which regions of any given sequence related to exons 2 and 3 and intron 2. After correctly arranging sequences in following orientation [exon 2 – intron 2 – exon 3], sequences were assembled from individual animals with a sequence representing the exon 2 derived peptide-binding region (PBR) of the black robin (*Petroica traversi*) (Miller & Lambert, 2004a). Each sequence was then translated into amino acids using Transform (M. Winter, 2009) and collated using Word (Microsoft). Separately untranslated sequences were collated in Notepad (Microsoft) TXT file using FASTA format. Individual sequences were denoted as 'alleles' with no consideration for specific loci. Duplicate sequences were searched for using the 'Find' function in Word and Notepad and the translated sequences were screened manually for anomalies (e.g. unexpected amino acids not fitting observed pattern of conserved regions of the PBR) and errors were augmented in the raw sequence data in CodonCode Aligner. Subdomains of the PBR were assigned based on She *et al.* (1991) and Miller & Lambert (2004a). This process was repeated for the exon 3 region, which was annotated using the common raven (*Corvus corax*) exon 3 sequence (Canal *et al.*, 2010). To establish the likelihood of the given sample size of sampling, most or all of the available diversity in each region of the MHC Class II B allele discovery curves were constructed for both exons in Excel (Microsoft). A maximum-likelihood phylogenetic tree was computed in CodonCode and edited in

MEGA version 5.1 (Tamura *et al.*, 2011) and was used to establish the phylogenetic relationship of the NSB MHC Class II B PBR and other species of bird, with the little spotted kiwi (*Apteryx owenii*) as the out-group (as a ratite it is therefore basal in the context of avian evolution). This ensured that the NSB sequences were appropriately placed within the tree (i.e. there is no evidence of contamination from other taxa, including *Homo sapiens*). This tree also included three other non-passerine bird taxa (Genbank Accession Numbers given in parantheses): *Gallus gallus* (chicken/red jungefowl) (EU502870), *Falco peregrinus* (peregrine falcon) (JN613255) & *Bubo bubo* (European eagle owl) (EF370932) and six passerines: *Petroica australis* (New Zealand robin) (AY730421), *Agelaius phoeniceus* (red-winged blackbird) (AF030988), *Geothlypis trichas* (common yellowthroat) (JX213842), *Acrocephalus arundinaceus* (great reed warbler) (AJ404372), *Andropadus virens* (little greenbul) (AY437898) & *Luscinia svecica* (bluethroat) (HQ539613). These sequences were selected to provide a broad range of avian taxa from some of the most ancient lineages (kiwi) to some of the more modern groups (passerines), so as to give context to where the NSB fits into the tree. All sequences were extracted from the NCBI nucleotide database.

As per Hughes & Nei (1989), evidence of balancing selection was inferred from an estimation of the ratio of non-synonymous (d_N) > synonymous (d_S) nucleotide substitutions in coding regions. Non-synonymous substitutions represent mutations that may have influenced the phenotypic trait (in this case the immunity to a particular pathogen) and will therefore be under selection. If balancing selection is occurring, proportionally more non-synonymous substitutions than synonymous substitutions would be expected. Using nucleotide sequence data in FASTA format, MEGA was used to calculate an estimate of $d_N : d_S$ ratios for both exon 2 and exon 3 regions using the Z-test of Selection (implementing the Nei-Gojobori method (with Jukes-Cantor correction) bootstrapped across 10,000 replications, enabling a direct comparison between regions. *p* values (<0.05) for whether the values are significantly different from 1 were calculated using a test hypothesis of Positive Selection ($d_N > d_S$) (i.e. one-tailed test). This method is used in a number of similar studies of MHC diversity and evolution (e.g. Jarvi *et* al., 2004; Miller & Lambert, 2004; Zhang *et al.*, 2006; Eimes *et al.*, 2010) but Kryazhimskiy & Plotkin (2008) have cast doubt on the reliability of this method for inferring selection mechanisms,

especially for single species samples. Therefore, while the $d_N : d_S$ may be indicative of the type of selection, caution should be used when intepreting the results for intraspecific samples.

To help clarify the relationship between individual MHC exon 2 sequences and to establish the number of different loci, phylogenetic trees were constructed using Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP) methods in MEGA. For the ML tree, the best model was determined using the Model Selection function in MEGA, which ranks model parameter sets in order of suitability for a given dataset, based on goodness of fit to model parameters. ML tree was constructed using the Jones-Taylor-Thornton (JTT) model with Gamma distribution with invariant rates among sites (5 discrete categories) and Nearest-Neighbor-Interchange heuristic method. NJ tree was constructed using the JTT model with Gamma distribution (Gamma parameter = 0.25). MP tree was constructed using a Subtree-Pruning-Regrafting search method with an initial number of 10 trees. Each of the three models was tested by bootstrapping (1000 replications). Sequences were putatively assigned to loci based on branches with high bootstrap values (>65) and level of conservation between all three trees, following Sato *et al.* (2011). Sequences designated as putatively functional alleles were restricted to complete sequences without deletions, frame-shift mutations or stop codons. Chicken (*Gallus gallus*) exon 2 sequence was used to root all three trees (Genbank Accession Number EU502870).

The 454 pyro-sequencing data obtained from a single NSB (01M04) in Chapter 3 (for the intial purpose of microsatellite discovery) was screened for MHC Class II B sequences in birds of the Order Passeriformes using the BLAST search of the NCBI nucleotide database http://www.ncbi.nlm.nih.gov/nuccore (3.2.6) and an analysis of these sequences was performed as per sequences obtained by cloning.

Finally, in order to elucidate any correlations between introns and exons, a selection of 45 intronic sequences from nine individuals were analysed. Alignment of introns was performed in CodonCode Aligner and ML and NJ Trees constructed to show sequence clusters. Sequences were rooted with an intron 2 sequence outgroup from the least auklet (*Aethia pusilla*) (Genbank Accession Number AY327422.1). These sequence clusters were compared with the results of the ML tree constructed for exon 2 and any possible relationships between clustering patterns inferred.

5.3 Results

A total of 383 usable sequences of the exon 2 region (249 bp) of the MHC Class II B were generated for the 30 NSBs sampled, yielding approximately 260 distinct alleles, of which at least 44 appeared to be non-functional (Tables 5.2 & 5.4). Of the non-functional alleles, 14 contained premature stop codons and 31 had identifiable frame-shift mutation (30 of which contained the same deletion of nine bases representing the absence of tyrosine/valine/histidine residues). This extraordinary diversity in the exon 2 region was not observed in the 222 sequences obtained for the exon 3 region (261bp), which only represented 29 distinct alleles, three of which contained a premature stop (Table 5.3). Therefore, MHC Class II B exon 3 in the NSB appeared to be considerably more conserved than exon 2, which is highly polymorphic and showing extensive sequence variation.

Comparison of sequences obtained for the same individual (07M05) from amplification by 'standard' (MyTaq) and 'high-fidelity' Taq polymerases showed no difference in error rate. Moreover, sequence errors appeared to be largely arising from the sequencing process (e.g. small or overlapping peaks on the sequencing chromatogram). Furthermore, no identifiable chimeras were observed in alignments for exon 2 and while one possible chimeric sequence was observed for exon 3 (NSBEX3-29), this sequence showed enough similarity to the other 28 sequences that it may possibly be a genuine exon 3 sequence.

Allele discovery curves were constructed for sequences from both exon 2 and exon 3 (Figure 5.1), based on random sampling within the sample of sequenced alleles, and the steep gradient of the curve indicates that we have only sampled a fraction of the available genetic diversity for exon 2. In contrast, the curve for exon 3 reaches a strong plateau within the given sample size of 222 sequences, indicating that much of the diversity within this region of the MHC Class II B has been captured. In addition, it strongly underlines the differential levels of variation occurring between these two exons.

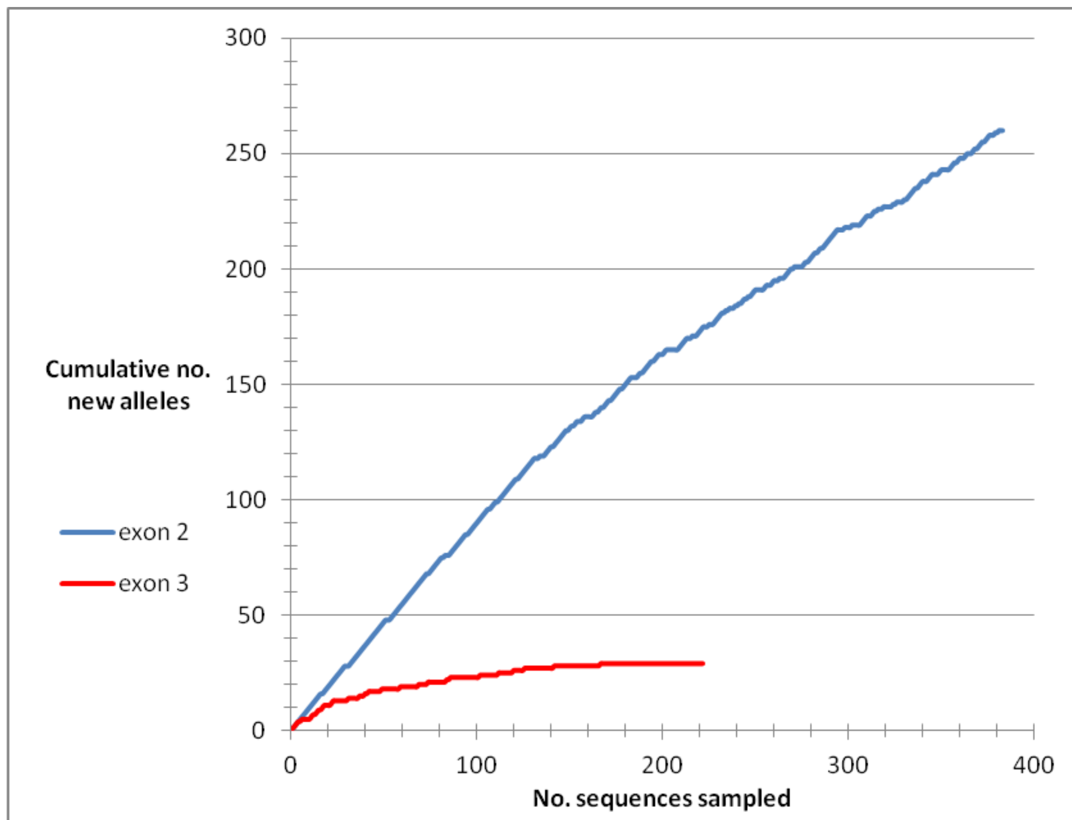


Figure 5.1 Allele discovery curve for exon 2 and exon 3 of MHC Class II B from sequences (exon 2 *n* = 383; exon 3 *n* = 222) obtained from 30 individual noisy scrub-birds (ten from each of the MG/MP/BA demes). x and y values calculated by Excel (Microsoft).

Table 5.2 Unique amino acid sequences obtained for exon 2 of the MHC Class II B in the NSB: (a) Presumed functional sequences (n = 214); (b) sequences containing stop codons (n = 14); (c) sequences containing frame-shift mutations (n = 31)

a)

Atcl01	KGQCYFTNGNEQVRFVQRYFYNRKQLLHFDSDGGHYVGDTPFGEDSGGNWNSKPELLEDRRAQVDTFCRHNYEVSTLFLVDP
Atcl02	------M---
Atcl46	------S---
Atcl60	-----Y--
Atcl71	------I---
Atcl80	-----YI---
Atcl82	-----YR--R--------------
Atcl94	-----YM---
Atcl121	-----YS---
Atcl130	------M---L---------------
Atcl154	-----YR---
Atcl228	------R---
Atcl06	--E---R---
Atcl32	--E---M---
Atcl36	--E---S---
Atcl38	--E---
Atcl40	--E---I---
Atcl58	--E--YS---
Atcl69	--E--Y--
Atcl131	--E--YM------------------------------------K--------------------------------------
Atcl168	--E--Y---R------------
Atcl182	--E---M---Q------------L
Atcl209	--E--R--------------
Atcl243	--E--YM----------------------N--
Atcl248	--E--YR---
Atcl251	--E--Y---G------------------------------
Atcl12	-A--

```
Atcl103 -A---Y----------------------------------------------------------------------------
Atcl155 -A---YM---------------------------------------------------------------------------
Atcl196 -A----S-----------H---------------------------------------------------------------
Atcl219 -A--------------D--I---E-F--L-----------------------------------------------------
Atcl250 -A----M---------------------------------------------------------------------------
Atcl09  -AE--YI---------------------------------------------------------------------------
Atcl42  -AE---I---------------------------------------------------------------------------
Atcl43  -AE--YM---------------------------------------------------------------------------
Atcl83  -AE---M---------------------------------------------------------------------------
Atcl102 -AE---S---------------------------------------------------------------------------
Atcl150 -AE--YS---------------------------------------------------------------------------
Atcl192 -AE-------------------------------------------------------------------------------
Atcl247 -AE---M-----------------------------------------------------------------K---------
Atcl249 -AE---R---------------------------------------------------------------------------
Atcl101 -V---YR---------------------------------------------------------------------------
Atcl181 -V---YI---------------------------------------------------------------------------
Atcl215 -V----M---------------------------------------------------------------------------
Atcl216 -V---YS---------------------------------------------------------------------------
Atcl04  -VE---S---------------------------------------------------------------------------
Atcl05  -VE--YI---------------------------------------------------------------------------
Atcl117 -VE--YM---------------------------------------------------------------------------
Atcl152 -VE---R---------------------------------------------------------------------------
Atcl153 -VE--YR---------------------------------------------------------------------------
Atcl165 -VE--YS---------------------------------------------------------------------------
Atcl15  -----Y---I-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl22  ---------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl51  ------S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl54  -----Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl96  ------M--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl98  -----------R----D--I-D-E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
```

```
Atcl118 -----YR--T-R----D--I---E--------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl123 ------I--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl145 ------I--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl161 ------R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl164 ------S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl176 -----YR--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IHR-R----FRP-S--R
Atcl177 ------M--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl178 -----YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl184 -----YM--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl197 -----Y---I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl202 ------I--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl206 -----YI--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl229 -----Y----------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl231 -----YI---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl236 -----Y---T-R--L-D--I---E-F------V------A--R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl260 -----Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IHR-R----FRP-S--R
Atcl03  --E---S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl17  --E--YI--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl28  --E--YI--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl31  --E--YR--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl45  --E---R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl47  --E---R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl48  --E--Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl52  --E--YI-------L-D--I---E-F------V--------LR-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl53  --E--YM--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl55  --E------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl62  --E---S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl65  --E--YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IHR-R----FRP-S--R
Atcl87  --E---M--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl107 --E--YS----P----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
```

```
Atcl136 --E--YR--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl138 --E--Y---I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH-------FRP-S--R
Atcl158 --E--Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IHR-R-C-MFRP-S--R
Atcl166 --E--YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl171 --E--Y------------------------------------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl203 --E--YR---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl211 --E--YI---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl224 --E--YI--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl226 --E---R--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl233 --E------I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl235 --E------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl259 --E--Y---I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl16  -A---Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl24  -A---YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl37  -A----S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl49  -A----M--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl63  -A----R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl81  -A---Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl105 -A---YI--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl122 -A---YR--T-R-K--D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl128 -A---Y---I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl186 -A---YR--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl217 -A-------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl220 -A---YR--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl35  -AE---R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl50  -AE---R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl89  -AE------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl97  -AE---S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R-H--FRP-S--R
Atcl111 -AE------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----LRP-S--R
Atcl135 -AE---M---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
```

```
Atcl162 -AE--YS--T-R----D--I---E-F---H--V-----V---R-IKAKRF--D--I--HE-SA--IHR-R----FRP-S--R
Atcl173 -AE--YM--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl175 -AE---M--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl180 -AE---M--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl185 -AE---S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl191 -AE--Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl194 -AE--YS---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl200 -AE--YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl223 -AE---S--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl227 -AE---I--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IHR-R----FRP-S--R
Atcl245 -AE---S--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HELSA--IH--R----FRP-S--R
Atcl253 -AE---S---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl91  -V----R---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl159 -V-------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl187 -V---YR---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl204 -V----R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl26  -VE---I---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl30  -VE---R---------D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl74  -VE--Y---T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl108 -VE--YS--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl112 -VE---R--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl133 -VE--YI--T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl139 -VE---S--I-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl146 -VE------T-R----D--I---E-F------V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl08  -----YI--T-R--L-E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl67  ------R--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl85  ------I--T-R--L-E--I---G-YA-----V---E--A----IQARY---Q--I--N-------V-------I-P-S--R
Atcl205 ---------T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---VR------FRP-S--R
Atcl13  --E---I--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl18  --E--YR--T-R----E--I---G-YV-----V---E--A----IQARY---Q--I----------V-------I-P-S--R
```

```
Atcl29  --E---S--T-R----E--I---G-YV-----V---E--A----IQARY---Q--I----------V-------I-P-S--R
Atcl34  --E---M--T-R----E--I---G-YV-----V---E--A----IQARY---Q--I----------V-------I-P-S--R
Atcl59  --E------T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl66  --E--YI--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl77  --E------T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl104 --E--YI--T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y-------FRP-N--R
Atcl124 --E------T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl125 --E---I--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl137 --E--Y---T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl201 --E---R--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl232 --E--YS--T-R----E--I---G-YV-----V---E--A----IQARY---Q--I----------V-------I-P-S--R
Atcl258 --E---M--T-R----E-H----G--------V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl129 -A----M--T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl174 -A---Y---T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl242 -A-------T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl41  -AE------T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl72  -AE--YR--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl93  -AE---S--T-R----E-H----G--------V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl126 -AE---I--T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl179 -AE---R--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl207 -AE---S--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl252 -AE--YI--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl14  -V---YI--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl113 -V-------T-R--L-E-H----G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl115 -V----R--T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y--------RP-S--R
Atcl172 -V----R--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl246 -V----S--T-R----E--IH--G-YA-----V---E--A----IQARY---Q--I--RK--A---Y-------FRP-S--R
Atcl11  -VE---I--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl33  -VE--YR--T-R----E--I---G-YV-----V---E--A----IQARY---Q--I----------V-------I-P-S--R
Atcl61  -VE---S--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
```

```
Atcl76  -VE--YM--T-R----E--I---G-YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl79  -VE--YI--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---V------GI-P-S--R
Atcl119 -VE---I--T-R--L-E-H-H--G-YV-----V---E--A-Y--IQARY---Q-----N---E---V-------FRP-S--R
Atcl144 -VE--Y---T-R----E--I---G-YA-----V---E--A----IQARY---Q--I--RK--A---Y--R----FRP-S--R
Atcl167 -VE--YR--T-R--L-E-H----G-YV-----V---E--A-Y--IQARY---Q-----N---E---A------GI-P-S--R
Atcl188 -VE---R--T-R--L-E--I---G-YA-----V---E--A----IQARY---Q--I--N-------V-------I-P-S--R
Atcl213 --E--YS--T-KI-LLA-AM---E--------V-LF-----W--KQARYY--Q--IM-YN-GI---L-----G-YAP-IA-H
Atcl214 --E---R--T-KM-L-A-AM---E--------V-LF-----W--KQARYY--Q--I--YN-GI---L-----G-YAP-IA-R
Atcl151 -----Y---MDW---ME-HI---E-------YV---L---LY--KVAKYL--RL-WM-YKGTV--RH-----KLDA---M-C
Atcl99  --E--Y---MDW--LME-H----E-------YV---L---LY--KVAKYL--RL-WM-YKGTE--RH-----KLDA---M-C
Atcl257 --E--Y---M-W--L-E-H----E-------YV---E---LY--KVAKYL--QL-WM-NKGTE--RH-----KLV---IM-C
Atcl84  -AE---R--MDW---ME-HI---E-------YV---L---LY--KVAKYL--RL-WM-YKGTV--RH-----KLDA---M-C
Atcl27  -----YR--T-R--L-E-LI---G-YA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl149 ------I--T-R--L-E-H----G-YV-----V---E--A-Y--KVARY---Q-----N---E---V-------FRP-S--R
Atcl170 ------S--T-R----E-H----G-YV-----V---E----Y--KVARY---Q--I--N-------V-------I-P-S--R
Atcl190 ---------T-R----E-H----G-YV-----V---E----Y--KVARY---Q--I--N-------V-------I-P-S--R
Atcl198 ------S--T-R--Y-E-LI---G-YV-----V---E--A-Y--KVARY---Q---------T---Y---------P----R
Atcl238 ------R--T-R--L-E-LI---G-YA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl254 -----YS--T-R--L-E-LI---G-YA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl64  --E---M--T-R--L-V--I---G-YA-----V---E--A-Y--KVARY---Q--I--N---E---V------L--P----R
Atcl68  --E---I--T-R--L-V--I---G-YA-----V---E--A-Y--KVARY---Q--I--N---E---V------L--P----R
Atcl86  --E--YR--T-R--L-V--I---G-YA-----V---E--A-Y--KVARY---Q--I--N---E---V------L--P----R
Atcl106 --E---S--T-R--L-E-LI---G-YA--H--V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl109 --E---S--T-R--L-E-LI---G-YA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl183 --E------T-R--Y-E-LI---G-YV-----V---E--A-Y--KVARY---Q---------T---Y---------P--M-R
Atcl193 --E---R--T-R----E-H----G-YV-----V---E----Y--KVARY---Q--I--N-------V-------I-P-S--R
Atcl199 --E--YM--T-R--Y-E-LI---G-YV-----V---E--A-Y--KVARY---Q---------T---Y---------P----R
Atcl237 --E--YS--T-R--Y-E-LI---E-Q------V---E--A-Y--KVARY---Q---------T---Y------GI-P----R
Atcl10  -A----I--T-R--Y-E-LI---E-Q------V---E--A-Y--KVARY---Q---------T-G-Y------GI-P----R
Atcl163 -A---Y---T-----LD-FI---E-YVM----L--F--F--N--KVARY---N-----RY-NG---Y--Q---ALSP-SLER
```

```
Atcl25  -AE---I--T-R--Y-E-LI---E-Q------V---E--A----KVARY---Q---------T---Y---------P----R
Atcl88  -AE---R--T-R--L-VK-I---G-YA-----V---E--A-Y--KVARY---Q--I--N---E---V------L--P----R
Atcl110 -AE---R--T-R--L-V--I---G-YA-----V---E--A-Y--KVARY---Q--I--N---E---V------L--P----R
Atcl127 -AE--Y---T-R--Y-E-LI---E-Q------V---E--A-Y--KVARY---Q---------T---Y------GI-P----R
Atcl189 -AE---S--T-R--L-E-LI---G-YA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
Atcl95  -VE---M--T-R--Y-E-LI-D-E-Q------V---E--A-Y--KVARY---Q---------T---Y------GI-P----R
Atcl255 -VE--YR--T-R--L-E-LI---GRYA-----V---E--A-Y--KVARY---Q-----N---A---Y-------I-P-S--R
```

b)

```
Atcl56  -A----R--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl57  ------M--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl70  --E--Y---TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl92  -AE---S--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl116 -VE--Y---TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M-IP----R
Atcl120 -A---YS------------------------------------------•--------------------------------
Atcl134 -A---YR--T-R----E--I---G•YA-----V---E--A-Y--IQARY---Q---------A---Y---------P----R
Atcl141 --E---I--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl143 -----YI--TDR------•----E•Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl156 -A----R------G-----------------------------------•--------------------------------
Atcl208 -AE---M--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl221 -V----I--TDR------•----E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl222 --E--YS--TDR------•S---E-Q------V-----------I-AR----D--I---M--R-----LY---M--P----R
Atcl256 -A-------TDR------•----E-Q------V-----------I-AR----D--I------R-----LY---M--P----R
```

c)

```
Atcl07  -AE--YR--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl19  -VE--Y---T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl20  -----YI--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl21  --E---I--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
```

```
Atcl23  --E---S--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl39  --E---R--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl44  -A---YS--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl73  ------I--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl75  -A----I--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl78  ---------T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl90  -A-------T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl100 ///---M--T-R--L-E--I---G-YA-----V---E--A----IQARY---Q--I--N-------V-------I-P-S--R
Atcl132 -----YR--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl140 -AE---I--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl142 -AE---M--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl147 --E--YI--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y-------FRP-S--R
Atcl148 -A---YM--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl157 -A---YR--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl160 ------R--T-R--YM-------E-///----V---------R-IKAKRF--D--I--HE-SA--IH--R----FRP-S--R
Atcl169 -A----S--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl195 --E--YR--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl210 -A---Y---T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl212 -AE--Y---T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl218 -VE---I--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl225 -VE--YS--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl230 --E---M--T-R--YM-------E-///----V--------Y-GINVR----DA-R------S-H-Y------LC-P-S-EH
Atcl234 ---------T-RM-YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl239 -AE--YS--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl240 -----YS--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl241 -AE--YS-------YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
Atcl244 -V---YI--T-R--YM-------E-///----V--------Y--INVR----DA-R------S-H-Y------LC-P-S-EH
```

− residue identical to reference sequence; • stop codon; /// location of missing amino acids due to frame-shift mutation

Table 5.3 Unique amino acid sequences obtained for exon 3 of the MHC Class II B in the NSB: (a) Presumed functional sequences (*n* = 26); (b) sequences containing stop codons (*n* = 3)

a)

NSBEX3-1	PPSPSQSIPSPSPCHTSPPTCLPVPPSVSISLVPSSSQPGPGRLLCPLMDFSPAQVQVRGFQGGRELTGHVVVTDVVPNRDWTYQVL
NSBEX3-3	--A------G-----L-
NSBEX3-4	---G-----L-
NSBEX3-5	---------------------------A---L-
NSBEX3-6	---L-
NSBEX3-7	--A------------L-
NSBEX3-8	---G-------
NSBEX3-9	--SV---Y---------------------------------L-
NSBEX3-10	--SV---Y----H---------------A------G-----L-
NSBEX3-11	--A------G-------
NSBEX3-12	-HR--HVS--APQLSPVS-RS--------V----------------SV---Y--P----W-------S-D--A------G-----L-
NSBEX3-13	-HR--HVS--APQLSPVS-RS--------V----------------SV---Y-------W-------S-D--A------G-------
NSBEX3-14	-HR--HVS--APQLSPVS-RS--------V----------------SV------P----W-------S-D--A------G-----L-
NSBEX3-15	-HR--HVS--APQLSPVS-RS--------V----------------SV---Y-------W-------S-D--A------G-----L-
NSBEX3-16	-HR--HVS--APQLSPVS-RS--------V----------------SV---Y--P----W-------S-D--A------G-------
NSBEX3-17	-HR--HVS--APQLSPVS-RS--------V----------------SV------P----W-------S-D--A------G-------
NSBEX3-18	-HR--HVS--APQLSPVS-RS--------V----------------SV-----------W-------S-D--A------G-------
NSBEX3-19	-HQSPHLS--APQLTPVS-RSI----G--G-------
NSBEX3-20	-HQSPHLS--AP-A-S-VSPLY-SA-G--G-------
NSBEX3-21	-HQSPHLS--AP-A-S-VSPLY-SA-G--G-----L-
NSBEX3-23	-HQSPHLS--AP-A-S-VSPLY-SA-G-------------------SV---Y--------------------A------G-------
NSBEX3-24	-HQSPHVS--AP-AQPGVSLLS-SA-QRVHLAGALELPAR-RPPAL--DG-L-CPGAGEAVP-RAGAH-ARGGHRRG-Q-GLDLPAA
NSBEX3-25	-HQSPHVS--AP-AQPGVSLLS-SA-QRVHLAGALELPAR-RPPAL--DG-L-CPGAGEAVP-RAGAH-ARGGHRRG-Q-GLDVPGA
NSBEX3-26	-HQSPHVS--AP-AQPGVSLLS-SA-QRVHLAGALELPAR-RPPAL--DG-L-CPGAGEAVP-RAGAH-ARGGHRRG-Q-GLDLPGA
NSBEX3-27	-HQSPHVS--AP-AQPGVSLLS-SA-QRVHLAGALELPAR-RPPAL--DG-L-CPGAGEAVP-RAGAH-ARGGHRRG-Q-GLDVPAA
NSBEX3-29	TSKAHAAP--SPQVIPVC-HS-----------L-----A--RG---SV---Y--HI-A-W---QQ--S----A------G-------

b)

```
NSBEX3-2   -HQSPHLS--APQLSPVSHRS-------------•----R--C---SV---Y--------------------A------G-----L-
NSBEX3-22  -HQSPHLS--APQLSPVSHRS-------------•----R--C---SV---Y--------------------A------G-------
NSBEX3-28  SQ-L-VHPQ-L-MP-Q--YMSPSA-QR-HLAGALELPARPRPPA-P-•WISR---------------------------------L-
```

• stop codon

Table 5.4 Comparison of sequences obtained for the exon 2 and exon 3 regions of the Noisy Scrub-bird MHC Class II B

Region	No. animals	No. sequences	No. alleles	No. functional alleles	d_N:d_S
EXON 2	30	383	260	218	1.685*
EXON 3	30	222	29	26	-1.040

d_N non-synonymous base substitution value; d_S synonymous base substitution value; * $p < 0.05$ calculated using Z-test of Selection (test hypothesis of Positive Selection ($d_N > d_S$)) in MEGA version 5.1 (Tamura *et al.*, 2011) based on bootstrapping across 10,000 replications (Nei-Gojobori method (Jukes-Cantor correction)

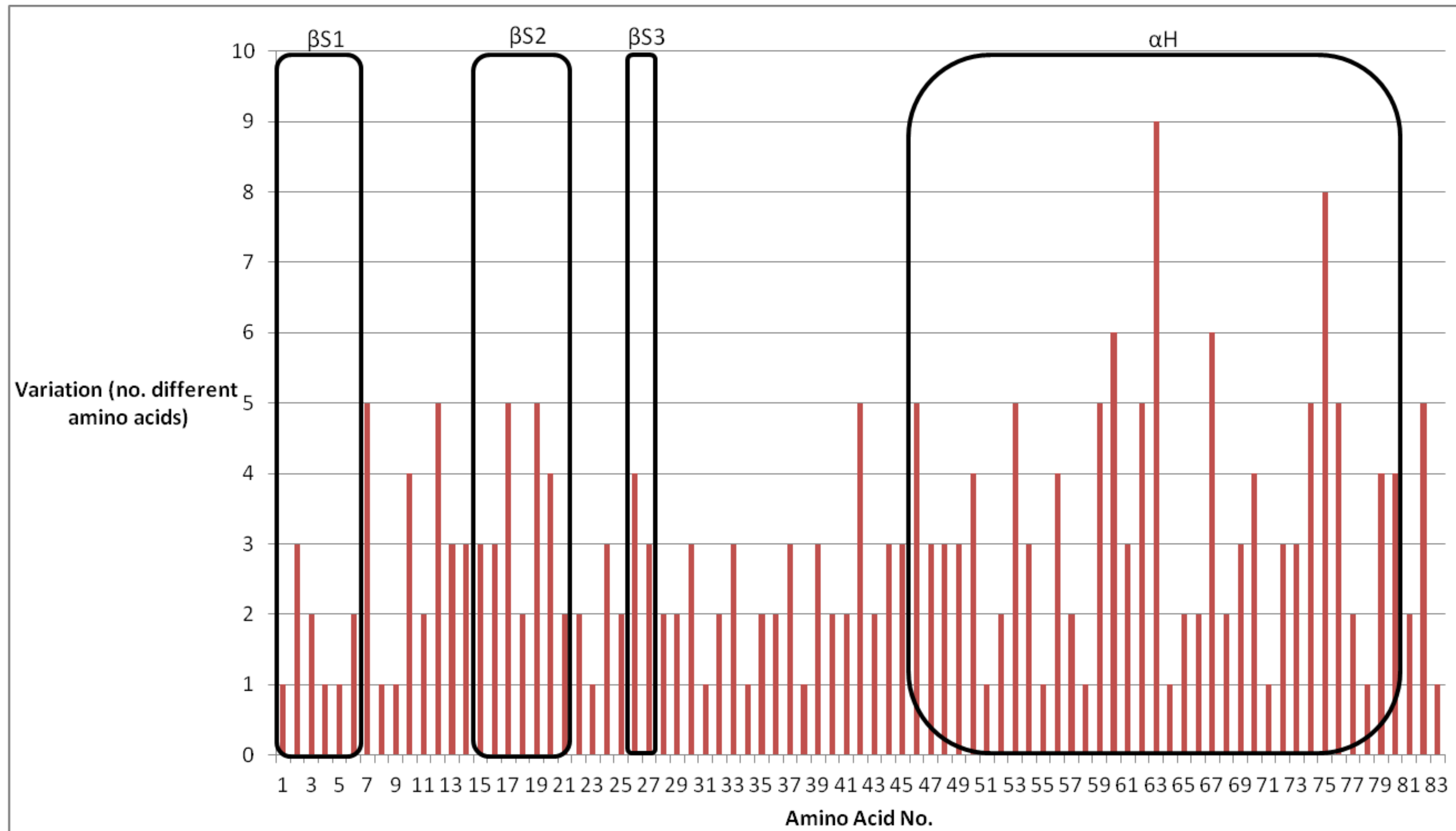


Figure 5.2 Histogram showing levels of variation for individual amino acids in the exon 2 region of the MHC Class II B in the NSB. Locations of β-sheet and α-helix domains are indicated by black boxes (βS - β-sheet; αH - α-helix) and were assigned based on She *et al.* (1991) and Miller & Lambert, (2004)

The value for $d_N : d_S$ for exon 2 was 1.685 and -1.040 for exon 3 (Table 5.4) but only the result for exon 2 was significantly different to 1 ($p < 0.05$).

A histogram of variation at individual amino acid residues was constructed for exon 2 to elucidate where the most variation exists in this region and which residues are the most conserved (Figure 5.2). This was annotated with the α-helix and β-sheet function domains (from She *et al.* (1991) and Miller & Lambert, (2004a)).

A phylogenetic tree for the PBR of 11 species of bird, including eight NSB sequences was constructed and the resulting tree is shown in Figure 5.3. All NSB sequences clustered together with the ratite out-group (*Apteryx owenii*), three non-passerine and six other passerine taxa in separate clusters, with the exception of little greenbul (*Andropadus virens*), which formed a discrete clade by itself. The pattern of clustering closely matches our existing knowledge of avian taxonomy, with the relatively ancient Atrichornidae family understood to be basal to the Australo-Papuan songbird radiation (Christidis & Norman, 2010).

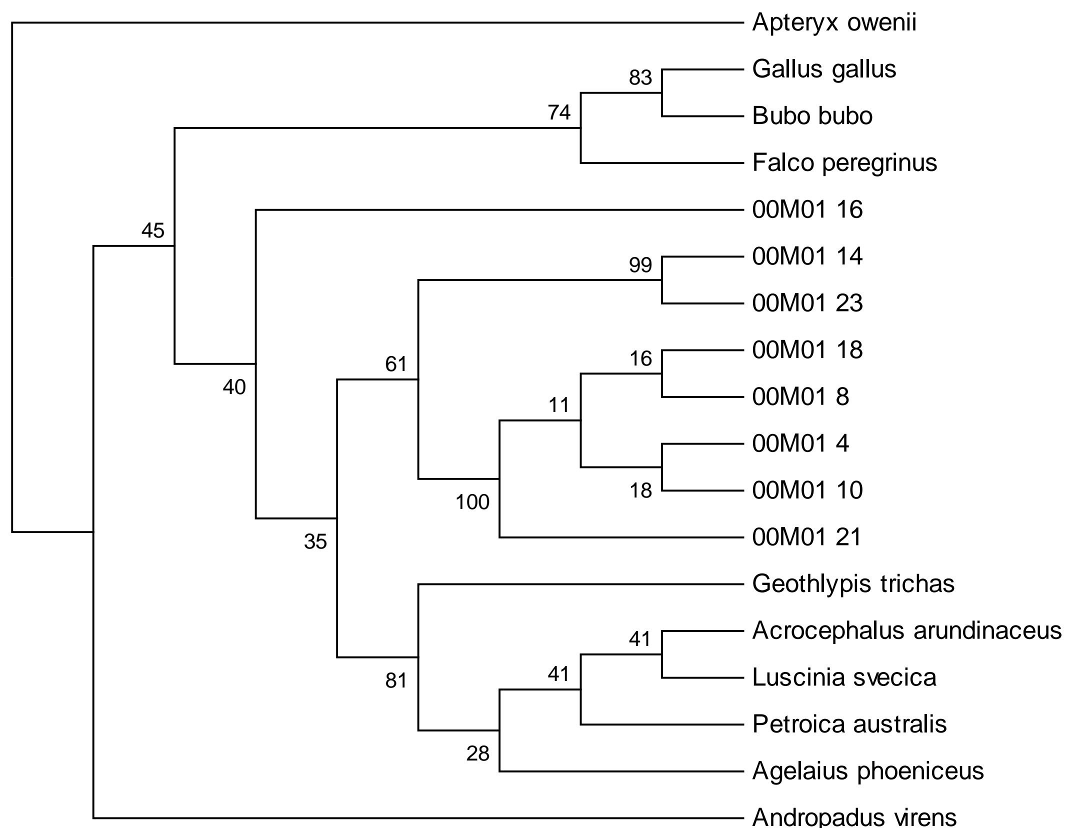


Figure 5.3 Phylogenetic tree for the peptide-binding region of the exon 2 of the MHC Class II B in eleven species of bird. 00M01 4 to 23 refer to NSB sequences from this study from the individual NSB 00M01. Bootstrap values are given at the base of each branch.

Screening of 454 pyro-sequencing reads for a single individual (01M04) by BLAST match of Passeriformes in the NCBI nucleotide database produced 62 strong matches for the MHC Class II B in 14 different species and 10 complete sequences (4 for exon 2; 6 for exon 3) were obtained from *Agelaius phoeniceus*, *Carpodacus mexicanus* (house finch), *Aphelocoma coerulescens* (Florida scrub-jay), *Geothlypis trichas*, *Petroica australis* and *Andropadus virens*.

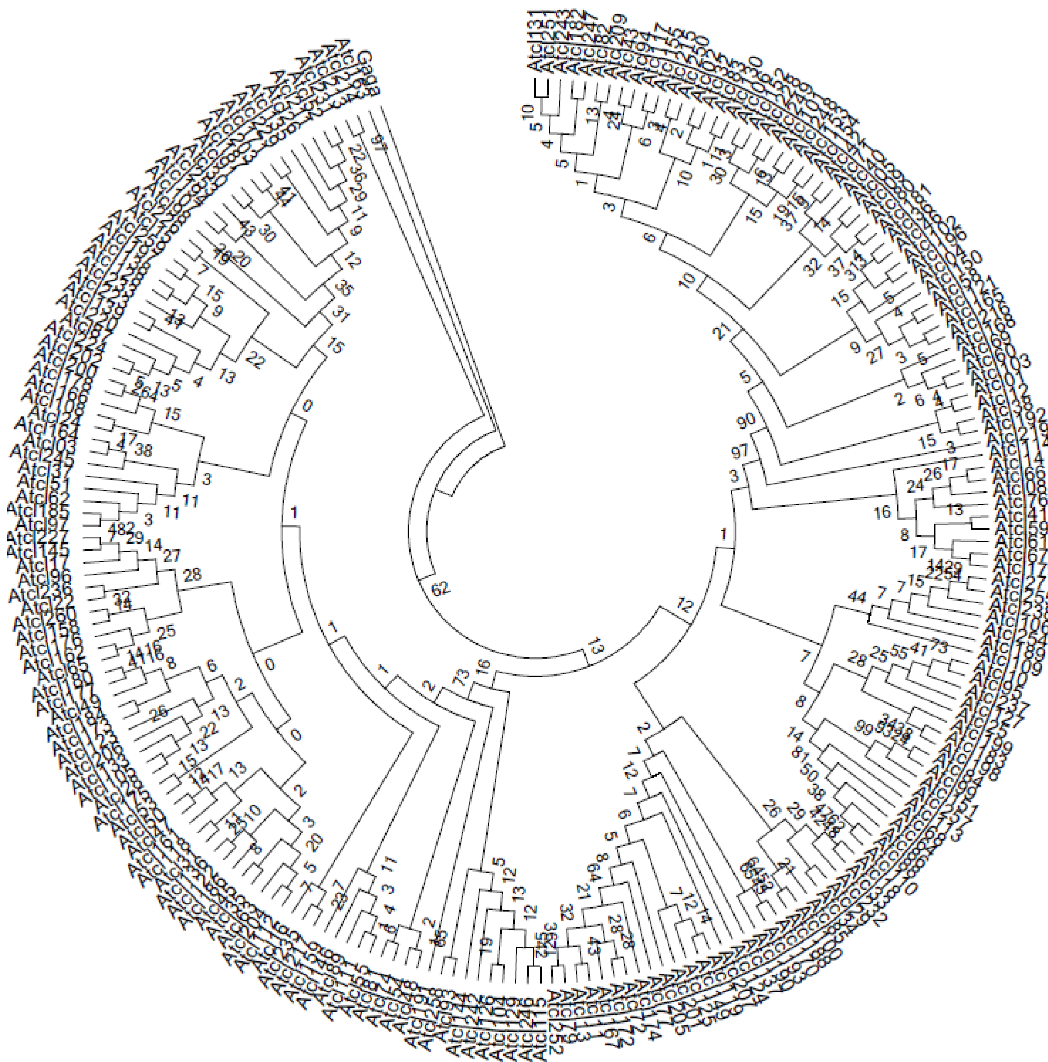


Figure 5.4 Example of phylogenetic tree (Neighbor-Joining) produced for putatively-functional sequences for exon 2 of MHC Class II B in NSB (rooted with chicken (*Gallus gallus* – Gaga).

The ML, NJ and MP trees constructed in MEGA were quite large, when the 216 putatively functional sequences were used (Figure 5.4). Bootstrap values and conservation between all three trees were used to putatively assign sequences to

specific loci (Table 5.5). Bootstrap values were highly variable, with branch values between 0 and 100, but many groupings were highly conserved between models, in spite of the apparently low bootstrap values. This may be coincidental or may reflect true relationships between branches. In total, sequences were assigned to 31 possible loci, with a minimum of 1 and maximum of 15 putative alleles per locus. The mean and median number of alleles per locus was 7.

Table 5.5 Locus assignment of putatively-functional exon 2 sequences based on clustering analysis (using Neighbor-Joining, Maximum Likelihood and Maximum Parsimony trees)

Locus no.	No. sequences assigned	Sequence number (Atcl_)
1	1	163
2	2	213, 214
3	4	84, 99, 151, 257
4	8	10, 25, 95, 127, 183, 198, 199, 237
5	7	27, 106, 109, 189, 238, 254, 255
6	6	64, 68, 86, 88, 110, 113
7	10	8, 14, 41, 59, 61, 66, 67, 76, 114, 179
8	3	170, 190, 193
9	8	119, 124, 125, 137, 149, 201, 205, 207
10	9	11, 13, 72, 77, 79, 167, 172, 174, 252
11	8	18, 29, 33, 34, 85, 100, 188, 232
12	9	93, 104, 115, 126, 129, 144, 242, 246, 258
13	10	4, 36, 46, 58, 102, 121, 150, 165, 196, 216
14	7	5, 9, 40, 42, 71, 80, 181
15	14	2, 32, 43, 83, 94, 117, 130, 131, 155, 182, 215, 243, 247, 250
16	9	1, 12, 38, 60, 69, 103, 168, 192, 251
17	1	219
18	15	26, 30, 52, 91, 98, 107, 135, 171, 187, 194, 203, 211, 229, 231, 253
19	5	128, 138, 197, 233, 259
20	5	24, 108, 166, 178, 200
21	8	3, 37, 51, 62, 97, 164, 185, 245
22	2	139, 223
23	2	15, 226
24	5	48, 54, 74, 81, 191
25	5	55, 89, 111, 146, 159
26	11	17, 22, 65, 96, 145, 158, 162, 176, 227, 236, 260
27	3	16, 217, 235
28	4	87, 202, 220, 224
29	12	28, 49, 53, 105, 123, 133, 173, 175, 177, 180, 184, 206
30	13	31, 35, 45, 47, 50, 63, 112, 118, 122, 136, 161, 186
31	10	6, 82, 101, 152, 153, 154, 209, 228, 248, 249

Figure 5.5 Maximum Likelihood tree for 45 intron 2 sequences in nine individual NSBs (Sequences assigned to specific putative loci numbers are highlighted as follows: 26 = yellow; 30 = red; 24 = light blue; 21 = light green; 20 = orange; 4 = saffron; 7 = dark green; 6 = dark blue; 13 = purple; 16 = brown)

Figure 5.5 shows a ML tree for the 45 intronic sequences sampled for clustering relationships. Based on the putative loci identified in Table 5.5, 11 clusters were identified within the intronic sequences tested. However, whilst some clusters appear to be conserved within branches (e.g, 'loci' 13, 26) other clusters are more widely spread across multiple branches (e.g. 'loci' 21, 30). However, bootstrap values vary and are as low as 29 and 33 for branches containing 'locus' 30 sequences. Branch patterns between ML and NJ trees were highly conserved with no significant differences in the distribution of sequences within the tree.

5.4 Discussion

5.4.1 Diversity in the MHC Class II B

In the field of conservation genetics, it is understood that, although neutral variation is useful for assessing the assessment of genetic diversity within and between populations (Holderegger *et al.*, 2006), as a predictor of evolutionary potential, adaptive variation may provide a much stronger tool (Hedrick, 2001). In a conservation context, evolutionary potential is very important since it helps to assess extinction risk (Frankham, 2005) and should be accounted for by conservation managers (Sgrò *et al.*, 2010).

In Chapter 4, levels of genetic diversity were evaluated at ten neutral (microsatellite) loci in three populations of the NSB and the results were complementary to what is known about the population history of the species. The microsatellite data exhibited low levels of allelic richness across all of the populations studied all of the populations studied (especially those established using small founder numbers) and inbreeding coefficients were high for two of the three populations. The microsatellite data exhibited low levels of allelic richness across all of the populations studied, and was especially so for populations established using small founder numbers and that inbreeding coefficients for up to two of the three populations were indicative of high levels of inbreeding. This was particularly evident in the parent population (MG), which is the only population not to have shown significant growth in recent years (Figure 1.5) and therefore inbreeding depression has been suggested as being a potential factor in the decline and stagnation of this population. However, to better understand the implications of this low diversity at neutral loci, it was desirable to establish levels of diversity using an adaptive gene group such as the classical MHC genes.

The findings using these genes are in striking contrast to the neutral locus diversity. From 383 sequences, across 30 individual NSBs, 260 different putative alleles were identified for the PBR of the exon 2 of MHC Class II B gene, of which 216 appear to be functional, although based on other studies it seems likely that many of these may be pseudogenes (e.g. Zagalska-Neubauer *et al.*, 2010). This level of diversity is unanticipated but not without precedent in birds. For instance, the common yellowthroat (*Geothlypis trichas*) was shown to have extraordinarily high levels of diversity for exon 2 in the MHC Class II B, with one individual alone yielding 39 different sequences (Bollmer *et al.*, 2010). In the collared flycatcher (*Ficedula hypoleuca*), 194 different alleles were sequenced from 237 individuals (Zagalska-Neubauer *et al.*, 2010). This is in contrast to other passerine bird species, such as the great reed warbler (*Acrocephalus arundinaceus*) (n = 7) (Westerdahl *et al.*, 2000), New Zealand robin (*Petroica australis*) (n = 41) (Miller & Lambert, 2004a) and house sparrow (*Passer domesticus*) (n = 13) (Bonneaud *et al.*, 2004). Moreover, all of these five species are widespread outbred species with little history of small populations and/or population bottlenecks. As discussed previously, population bottlenecks can result in reduction in genetic diversity within a species, including the MHC gene group. The prediction that there is low diversity in the NSB was based upon this theory as well as other examples of low levels of MHC diversity in bottlenecked bird species. However, it appears this hypothesis should be rejected and that the known population history of these three study populations of NSB makes the discovery of such extensive diversity even more remarkable.

Despite the surprising nature of these results, there is reason for high confidence in the data generated. Firstly, in the methods of amplification, ligation, transformation and subsequent analysis, every effort was made to minimise the impact of PCR or sequencing errors (including chimeras) on sequencing data. Secondly, most of the variation in the sequences is found in the areas of the gene that correspond with the antigen binding domains of the PBR (Figure 5.2). These data are very similar to what was observed in the New Zealand robin (Miller & Lambert, 2004a). The mean number of amino acids per site was 3.46, 3.43 and 3.50 for the β2- and β3-sheets and α-helix respectively, compared to 3.00, 2.00 and 2.33 for the inter-domain regions. Therefore, the likelihood that the results have arisen through PCR/sequencing errors can be considered to be low. Furthermore, patterns of variation are highly conserved, with motifs within the α-helix correlating with corresponding motifs in the β-sheet

domains (Table 5.2). Table 5.2 shows the complexity of the variation in exon 2 but also shows the conservation of particular motifs within the amino acid sequences. These motifs indicate that, whilst high levels of variation do exist in this gene, the variation itself is somewhat conserved to motifs within specific regions of the exon and is clearly not random. This underlines the likelihood that the observed variation is valid and not simply a result of PCR/sequencing errors or due to the formation of chimeras. Furthermore, additional support comes from the direct comparison between the variation observed in exon 2 and exon 3. Substantially more variation is observed in the exon 2 than the exon 3, and since the exon 2 codes for the functional region of the MHC Class II B (the PBR) in birds, it may be expected to accumulate more diversity than the exon 3 region. If some of the observed diversity in exon 2 is indeed erroneous, it would be expected that similar level of errors would also be present in the exon 3 region generating at least equivalent levels of allelic richness. However, based on allele discovery curves (Figure 5.1), the exon 2 appears to be an order of magnitude more diverse than exon 3. In addition, estimates of d_N : d_S indicate that the values for exon 2 are significantly greater than 1 but not for exon 3, indicating that balancing selection is occurring mainly within exon 2 (generating diversity for this region). Given the exon 2 codes for the functional region of the PBR, it might be expected for this region to be under greater selective pressure than the less important exon 3. Since under balancing selection, greater functional MHC variation will be retained than neutral variation after a bottleneck (Maruyama & Nei, 1981), it might be expected that in a bottlenecked species greater diversity would be maintained in exon 2 than in exon 3.

The possibility of contamination from gDNA from human or other taxa can be excluded, as maximum-likelihood tree in Figure 5.2 displays shows the NSB sequences sitting in the correct taxonomic position with respect to other avian PBR sequences, firmly placed between the non-passerine *Falco peregrinus* (perergrine falcon), *Bubo bubo* (European eagle owl) and *Gallus gallus* (chicken) and the rest of the passerines used in this tree.

Phylogenetic trees constructed using three different models (Maximum Likelihood, Neighbour-Joining and Maximum Parsimony) were used to assign sequences as alleles to putative loci. Bootstrap values between branches were occasionally very low and the dataset of 216 putatively functional alleles was quite unwieldy. Through identifying clusters of sequences conserved between the models, as per Sato *et al.*

(2010), loci could be assigned with a reasonably high level of confidence. So, whilst the designation of 31 loci for these 216 sequences may not be totally accurate, this figure remains informative for the purposes of this study. It has been shown that the common yellowthroat has a minimum of 20 loci encoding exon 2 of the MHC Class II B (Bollmer *et al*., 2010), indicating that the high number of loci estimated in this study is not entirely without precedent. A high number of loci with a relatively low number of alleles would potentially preserve more variation through a bottleneck and would facilitate rapid generation of post-bottleneck variation through mutation and gene conversion.

It was found in a small sample of 45 sequences from nine individuals that the clustering patterns observed within the exon 2 sequences corresponded to conserved intron 2 sequences. In mammals, intronic sequences have been used to infer loci but in passerine songbirds this is made more difficult by high levels of gene conversion (Miller & Lambert, 2004a). Gene conversion could result in many closely related loci, which may make introns a more unreliable method of predicting loci. However, the observed pattern of clustering provides some validation for the loci that were predicted in Table 5.5 as well as providing further independent evidence that the high number of exon 2 loci predicted is not due to artefacts.

Finally, BLAST results from the second-generation sequencing data obtained from a single NSB found a number of strong matches for the MHC Class II in a range of passerine bird species. These sequences were successfully aligned with sequences obtained by cloning but none of the 454 sequences matched these original sequences and it is unknown whether this relates to errors in the pyro-sequencing reads (which have been found to be in the order of around 1.07% but increasing up to 50% (Gilles *et al.*, 2011)) or whether they are indeed novel sequences. This potentially innovative approach to discovering MHC sequences from shotgun sequenced genomic data is limited by the issue of variable error rates in pyrosequencing and is therefore not a substiute for second-generation sequencing using tagged primers (e.g. Zagalska-Neubauer *et al*., 2010).

5.4.2 Implications for conservation

Given the crucial role the MHC plays in immunological function, diversity within this gene group may be extrapolated to infer levels of fitness within a population or species, although there may not always be a clear correlation (Radwan *et al.*, 2010)

and the role of QTLs in influencing MHC function should also be considered. Spielmann *et al.*, (2004) showed the role genetic diversity has on disease resistance in *Drosophila* spp. and the susceptibility of the species to various pathogens has been linked to MHC variation (O'Brien & Everman, 1988). Furthermore, there is some evidence for this link in vertebrates. For example, in the spotted suslik (*Spermophilus suslicus*) blood parasite loads are correlated with MHC diversity (Biedrzycka *et al.*, 2011). In birds, Westerdahl (2007) found that the MHC may also influence the prevalence of avian malaria in great reed warblers and house sparrows. Therefore, there is some existing evidence to suggest that MHC diversity does influence pathogen resistance (and therefore fitness), which in turn impacts on extinction risk. In this context, the extraordinary diversity of the PBR of the MHC Class II B in the NSB is a positive discovery. While there is evidence for an extensive loss of diversity at neutral loci, it appears that the NSB as a species has retained a large quantity of diversity at this adaptive locus. In comparison to other more genetically depauperate passerine species (e.g. black robin), the presented data suggest that the NSB is in better shape to respond to new pathogen challenges, although one caveat is that there is we have only a limited understanding of the functionality of these 216 alleles in which stop codons and frame-shift mutations are absent. However, the 216 sequences vs. the four observed in the black robin must certainly indicate that the NSB may be less vulnerable to the emergence of any new pathogen. This provides solace that extensive variation still exists in the NSB genome for traits that are related to fitness. Furthermore, the role of the MHC in reproduction and fecundity should also be considered, particularly when there is strong evidence for low MHC diversity inferring lower reproductive fitness, e.g. in European hares (Smith *et al.*, 2010b). Given that a) the high population growth on Bald Island and Mt Manypeaks (Figure 1.5) and b) the female NSB only lays a single egg, low fecundity is not a factor managers have had reason to be concerned about. Therefore, the high MHC diversity could be related to high fecundity (and hence breeding success) in these populations of which the opposite could be feared (based on the low neutral diversity discovered in Chapter 4). Additionally, the large number of loci indicated to be present for the MHC Class II B may provide evidence that the genome of the NSB is predisposed to help maintain variation in this gene group. However, the results for BA and MP are in contrast to MG, where the population has, in fact, decreased in recent years. However, if this decline is relating to genetic factors, it may not be

directly related to MHC-related traits such as fecundity or disease resistance, but may be due to inbreeding depression causing the recombination of deleterious alleles in other genes.

Nevertheless, the population history of the NSB must be considered, as the bottlenecks populations of this species have experienced may have reduced MHC diversity. Unfortunately, due to the large number of alleles and loci that apparently exist in the NSB genome, it was impossible to capture all available diversity in any single population, making inter-population comparisons impossible. Therefore, we are unable to draw conclusions on the impact of translocations of MHC diversity in the NSB. While there is evidence that pathogen-driven balancing selection can result in the maintenance of post-bottleneck MHC diversity (Hawley & Fleischer, 2012; Oliver & Piertney, 2012), it is conceivable that pre-bottleneck (i.e. historical) diversity of the MHC Class II B was even more extensive. Therefore, while managers should seek to maintain the unanticipated level of diversity remaining, consideration should also be given to the fact that some of the genetic variation lost in the bottleneck may be important for the fitness of the species. As such, the threat of new-and-emerging avian diseases (e.g. West Nile virus, avian influenza H5N1) must not be ignored regardless of the apparent robustness of the MHC variation in the NSB.

A question that stands out from these striking results is: how did this extraordinary diversity evolve in the first place? As previously discussed, it is understood that selection and the evolution of MHC variation is often driven by disease processes, as the immune systems seeks to keep pace with rapidly-evolving pathogens. This process has been shown to maintain MHC diversity even after a population bottleneck (in spite of a reduction in neutral diversity) in both the house finch (*Carpodacus mexicanus*) (Hawley & Fleischer, 2012) and water vole (*Arvicola terrestris*) (Oliver & Piertney, 2012). This possibly provides an explanation of how the NSB has maintained such high MHC diversity through at least one bottleneck. However, there is little contemporary evidence of significant pathogens in the NSB (Edmonds, 2007 unpublished) including no evidence of blood parasites. Whether this absence of pathogens (especially blood malarial parasites, as Adlard *et al*. (2004) estimated 11% prevalence across a range of Australian bird species) indicates a true absence in the environment or a high level of resistance is unclear. Therefore, gene conversion or another mechanism (e.g. behavioural), possibly in concert with

balancing selection, may be a possible driver of MHC diversity. To better understand the role of MHC diversity and general pathogen prevalence in birds, particularly island species, this study should be replicated in species that have higher pathogen loads than the NSB.

Chapter 6 – Diversity in Major Histocompatibility Complex Class II B gene and pathogen prevalence in two island bird species

The previous chapter described the identification of DNA sequences for the classical class II B genes [exon 2 - intron 2 - exon 3] derived from the Major Histocompatibility Complex (MHC) Class II region of the NSB genome and showed, quite unexpectedly, relatively high levels of sequence diversity across apparently multiple loci. To help elucidate if the observed diversity in this important immunological gene in the NSB is derived from pathogen-mediated balancing selection, these methods were replicated for two species of passerine birds from Barrow Island, Western Australia - the spinifexbird (SPB) and black-and-white fairy-wren (BWW) - and the work described in this chapter represents the first genetic diversity research performed on these species. Both these species have been shown to have a relatively high pathogen load and this has provided an opportunity to compare the relationship between pathogen prevalence and MHC in the species to be used as a possible model for populations of NSB. Diversity in the Class II B genes were observed to be lower in both species compared to the NSB with no clear evidence of balancing selection, pathogen-mediated or otherwise. However, while these results cannot eliminate a pathogen-mediated mechanism of evolution in the NSB, it suggests that another form of selection may be a more important driver of MHC evolution in this species. These results greatly enhance the understanding of the biology of all three species, two of which (NSB and BWW) are threatened taxa.

6.1 Introduction

As discussed in Chapter 5, there is some evidence that MHC diversity is important in ameliorating resistance to pathogens. In European rabbits (*Oryctolagus cuniculus*), the presence of a particular MHC haplotype has been shown to confer resistance to hepatic coccidia (Oppelt *et al.*, 2010) and similarly in water voles (*Arvicola terrestris*) heterozygous individuals for a particular MHC locus exhibit lower pathogen loads than homozygous individuals (Oliver *et al.*, 2009). However, in the lesser kestrel (*Falco naumanni*) there is no evidence of a correlation between MHC

diversity and avian malaria prevalence (Ortego *et al.*, 2007b) although in chickens (*Gallus gallus*) a strong association between MHC diversity and the prevalence of the mite *Ornithonyssus sylviarum* has been observed (Owen *et al.*, 2008). The MHC's role in promoting pathogen resistance is thought to be an important factor in the evolution of this gene group, as pathogen-driven balancing selection is thought to be a key driver increasing MHC diversity (Jeffery & Bangham, 2000). Furthermore, the MHC has been shown to sustain high degrees of polymorphism even in bottlenecked populations with relatively low levels of neutral locus diversity (Aguilar *et al.*, 2004; Hawley & Fleischer, 2012).

In Chapter 5, levels of diversity in the MHC Class II B gene group in the NSB were investigated, in both the exon 2 and exon 3 regions. It was shown that extraordinary levels of diversity existed for exon 2, which encodes for the peptide-binding region (PBR) of the mature MHC protein. However, considerably lower diversity was observed in the exon 3 region and this supports our understanding of the respective functionality of the peptides encoding regions of this gene. Furthermore, there was evidence of the presence of balancing selection in the PBR of the classical class II genes in the NSB, with a considerably higher proportion of non-synonymous base substitutions (d_N) to synonymous base-substitutions (d_S) observed. This was not observed for exon 3 region which showed an opposite ratio. Edmonds (2007, unpublished) found that the NSB appears to have an inherently low disease risk, with little or no evidence of blood or faecal parasites and although there was evidence of bacterial pathogens, no evidence of clinical disease was observed. However, it is unclear whether these results relate to a relatively low environmental pathogen prevalence or higher levels of innate immunity in the NSB. If the latter is the case, it would certainly bode well for the inherent immunity of the NSB in general and may help buffer any impact of the future emergence of new diseases. However, the observed lack of pathogens in the NSB may also relate to the bottleneck(s) the species has been exposed to, since the extinction risk of pathogens and their hosts are intrinsically linked. Studies on introduced species show that populations derived from small founder groups have a reduced probability of establishing a pathogen within that population (Torchin *et al.*, 2003), as in the case of the European starling (*Sturnus vulgaris*) in North America (Hair & Forrester, 1970), the which inherited just 11 of 70 helminths known from the Eastern Hemisphere, from which the 100 founder individuals originated from. This theory of 'parasite release' could be

extended to any population descended from a small founder group, e.g. a bottleneck or translocation.

However, to better understand the influence of MHC diversity on pathogen prevalence and as a possible model for NSBs populations, a study using two island bird species shown to have relatively high pathogen loads was undertaken. The SPB and BWW were translocated from Barrow Island (of the NW coast of Western Australia, Figure 1.4) to Hermite Island within the Montebello Group of islands 20-40km NNE of Barrow in 2010 and 2011 and in 2010 the translocated birds were screened for pathogens (A.H. Burbidge, *pers. comm.*). Approximately 23-32% of SPBs and BWWs screened were shown to be positive for *Chlamydophila psittaci* (Appendix iii), a gram-negative bacterium known to be endemic in some bird populations (although carriers may not always exhibit clinical signs of the disease) and while virulent strains may have a mortality rate of 5-30%, less virulent strains may have a mortality rate of < 5% (Pattison, 2008). The observed prevalence of *C. psittaci* in the SPB and BWW in itself is not necessarily significant given the high prevalence of this pathogen in some other bird species e.g. c.38-70% in tits (Paridae) (Holzinger-Umlauf *et al.*, 1997). However, given that these results are from an isolated island population, it is possible that *C. psittaci* is endemic in this population and there is potential that it may be driving the evolution of the MHC in both of these species. In this respect, these species may provide an analogue to the NSB, to test the hypothesis that pathogens (cryptic or otherwise) can drive evolution in the MHC Class II to the extent we see in the NSB. All three species are passerine birds that are either from island populations or (in the case of the NSB) have effectively been confined to 'islands' of suitable habitat (understood to reduce genetic diversity, as discussed in 1.2). However, pathology screening has failed to find any significant evidence of high pathogen prevalence, such as *C. psittaci* or coccidia (Edmonds, 2007 unpublished) in the NSB, both of which are found in SPBs and BWWs (Appendix iii). All three birds differ somewhat in their ecology (given that two are fundamentally arid species) but all are small passerines that generally live near or on the ground. It is known that, in mice, protective immunity to *C. trachomatis* and *C. muridarum* requires antigen presentation through the MHC class II presentation system (Karunakaran *et al.*, 2012).

In this study, the MHC Class II B exons 2 (coding for the PBR) and 3 were sequenced for the BWW and SPB in a replication of the methods used in Chapter 5,

to assess the relative levels of diversity in both of these regions in both species. Given the knowledge of pathogen-mediated MHC evolution, MHC diversity in the BWW and SPB is expected to be comparatively high in respect to other passerine birds species. Furthermore, it might be expected that levels of diversity in the SPB and BWW will exceed that observed for the relatively pathogen-free NSB. However, given that the sample size used was insufficient to capture all diversity within the exon 2 region for the NSB, this limitation will also be imposed on the other species preventing a direct comparison in the total numbers of alleles. However, given the margin by which the NSB MHC diversity exceeds other passerine birds, any comparable results for the BWW or SPB will be significant.

Additionally, the role of gene conversion and other behavioural mechanisms cannot be excluded and therefore the importance of other factors in generating MHC diversity was also investigated. PBR DNA sequence data from the NSB was also included in order to compare gene conversion in this species with the two Barrow Island species.

6.2 Methodology

Tissue samples (blood feathers) were taken from SPBs and BWWs that had been captured as part of the translocation from Barrow to Hermite Islands in 2010 and 2011 and DNA was extracted as per the protocol in 2.3.2. gDNA from 15 BWWs and 14 SPBs were used for amplification and sequencing. The methodology used for amplification and sequencing of the MHC Class II B exon 2 – intron 1 – exon 3 is described in 5.2.1, and was performed using the primer set P1/AlEx3R. In addition, the identification and augmentation of sequences was undertaken using the method described in 5.2.2.

Allele discovery curves were constructed in Excel (Microsoft) for both exons in both species and estimates of d_N:d_S were calculated using in MEGA version 5.1 (Tamura *et al.*, 2011). In addition, to ascertain the degree to which gene conversion is important in generating MHC diversity in these species, sequences were screened for evidence of this phenomenon using GENECONV 1.81 (Sawyer, 1999). GENECONV screens sequences for regions that share consecutive identical polymorphisms, which are of sufficient similarity to indicate gene conversion has occurred. Since coding sequences may show similarities due to the functionality of

specific polymorphisms, the software allows the option of screening ‘silent’ polymorphisms, that is polymorphisms in non-coding sequences. This method can be very useful in detecting gene conversions but is limited by performing poorly with highly divergent sequences (Drouin *et al*., 1999). Analyses were run with the gscale parameter set at 0 (no mismatches), 1 (few mismatches) and 2 (some mismatches) and with and without the ‘SILENT’ option (only analyses non-coding sequences).

6.3 Results

For the BWW, 206 exon 2 region sequences were obtained and 201 containing the exon 3 region (Table 6.2). From the exon 2 sequences, 87 different putative alleles were identified, of which no stop codons or frame-shift mutations were identified (i.e. all alleles appeared to be functional). From the exon 3 sequence, 16 putative alleles were obtained and again all appeared to be functional. For the SPB, 185 sequences for the exon 2 region were obtained and 165 for the exon 3 region (Table 6.2). The exon 2 sequences yielded 134 different putative alleles, of which 24 contained stop codons ($n = 20$) or frame-shift mutations ($n = 4$); exon 3 sequences yielded 48 putative alleles, of which three contained stop codons. These results are comparable with those observed for the NSB, with the proportion of exon 2 sequences being much higher than exon 3 sequences, the exon 3 being more highly conserved than the more polymorphic exon 2. However, unlike the NSB, the ratio of d_N:d_S for both Barrow Island species is very similar in both exon 2 and exon 3 and *p* values indicate that these values are not significantly different from 1. This is contrary to our expectations if balancing selection was acting only upon exon 2.

Allele discovery curves for the BWW (Figure 6.1) indicated that for both exon 2 and exon 3, our sample size has captured the majority of the available diversity in this population, as demonstrated by both curves forming clear plateaus. By contrast, the allele discovery curves for the SPB show that, while most of the available diversity for the exon 3 region appears to have been captured, considerable exon 2 diversity remains undiscovered. Regardless, the diversity that exists in both species (particularly for the exon 2 region) is far in excess of what might be expected in an

Table 6.2 Sequencing results for the exon 2 and exon 3 region of the MHC Class II B in the black-and-white fairy-wren (BWW) and spinifexbird (SPB) on Barrow Island

BWW

Region	No animals	No sequences	No alleles	No func. alleles	d_N:d_S
EXON 2	15	206	87	87	1.607
EXON 3	15	201	16	16	0.850

SPB

Region	No animals	No sequences	No alleles	No func. alleles	d_N:d_S
EXON 2	14	185	134	110	1.952
EXON 3	14	165	48	45	0.983

d_N, non-synonymous base substitution value; d_S, synonymous base substitution value; in MEGA version 5.1 (Tamura *et al.*, 2011) based on bootstrapping across 10,000 replications (Nei-Gojobori method (Jukes-Cantor correction)); no values for d_N:d_S have probability $p < 0.05$.

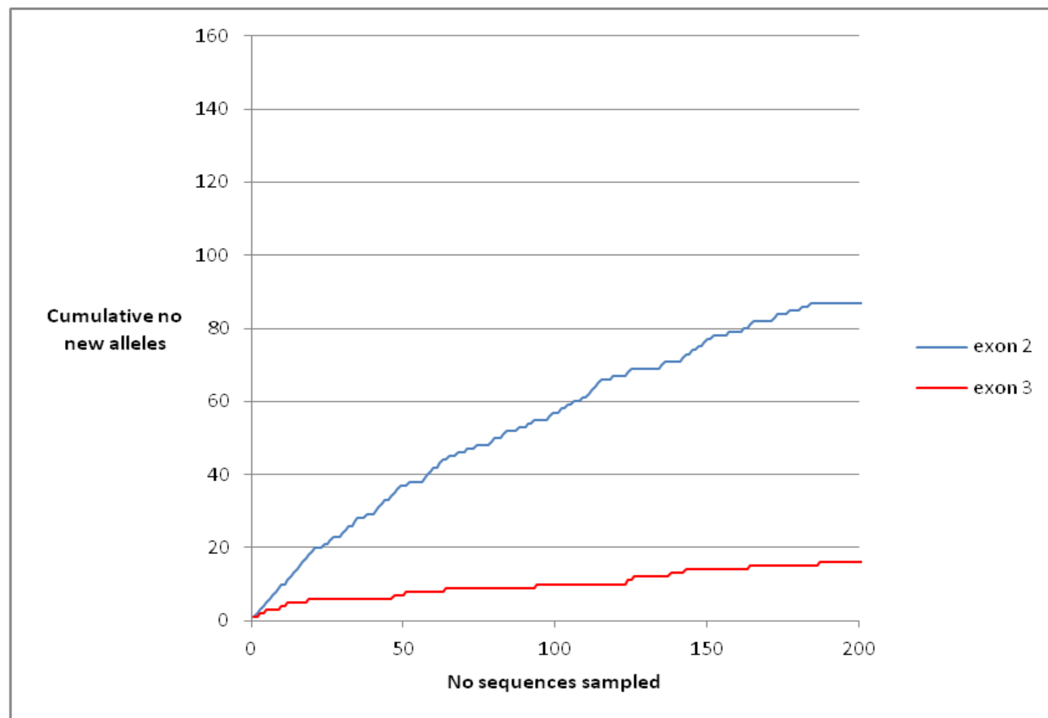


Figure 6.1 Allele discovery curve for exon 2 and exon 3 of MHC Class II B from sequences (exon 2 n = 206; exon 3 n = 201) obtained from 15 individual black-and-white fairy-wrens

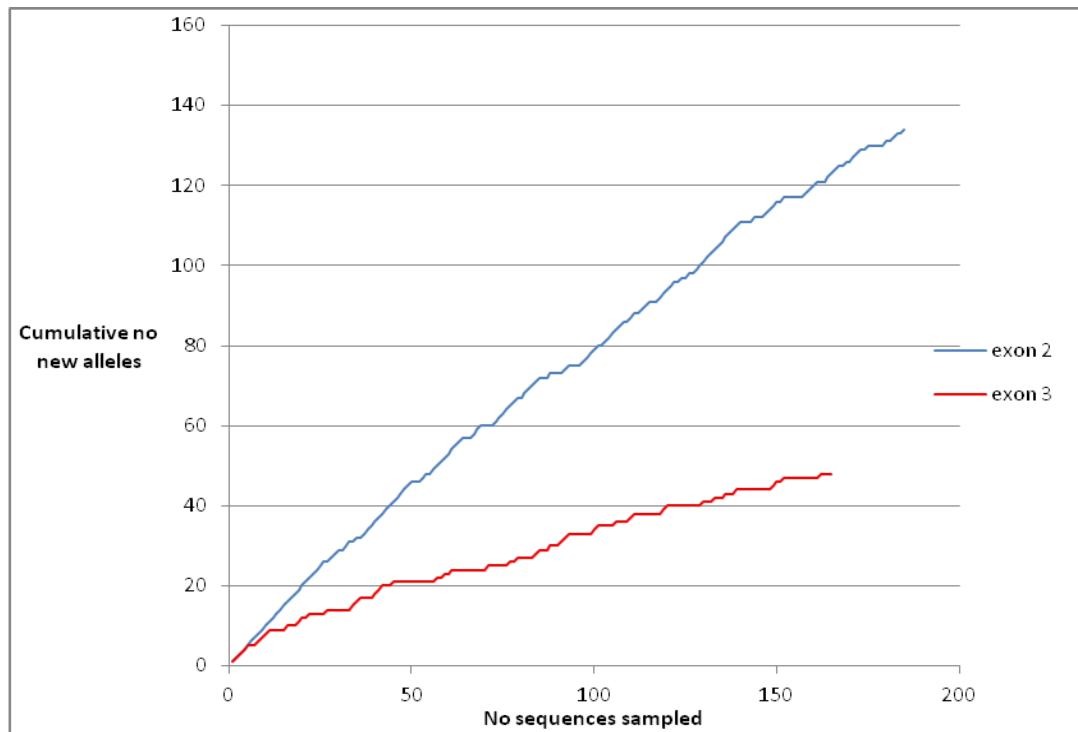


Figure 6.2 Allele discovery curve for exon 2 and exon 3 of MHC Class II B from sequences (exon 2 *n* = 185; exon 3 *n* = 165) obtained from 14 individual spinifexbirds

outbred passerine bird and, while the 87 BWW alleles are less than the >260 observed in the NSB, it is feasible that by sampling more individual SPBs, the number of different allele sequences could exceed that of the NSB.

Analysis with GENECONV for PBR sequences in BWW and SPB as well as the NSB (gscale = 0) found 149 putative gene conversion events in the BWW (from 206 sequences), 254 in the NSB from 260 sequences and only 2 in the SPB (Appendix ii). Using gscale = 1 produced zero conversions in BWW and SPB and gscale = 2 produced six and one respectively. For the NSB, gscale = 1 produced 190 postulated conversions and gscale = 2 produced 489. Since the default setting for GENECONV is gscale = 0 (i.e. excludes all mismatches) this was used as the most robust estimate. Repetition with the 'SILENT' option produced 0 results for all three species, which is what would be expected given that all sequences are from a coding region of the MHC.

Our confidence that the observed levels of diversity are a reflection of the population is high, using the same reasons given in 5.4. Variation in amino acids profiles mirrors that observed in the exon 2 in the NSB βS1 domain of the PBR. In the other domains, both BWW and SPB gene sequences vary in quite different ways when

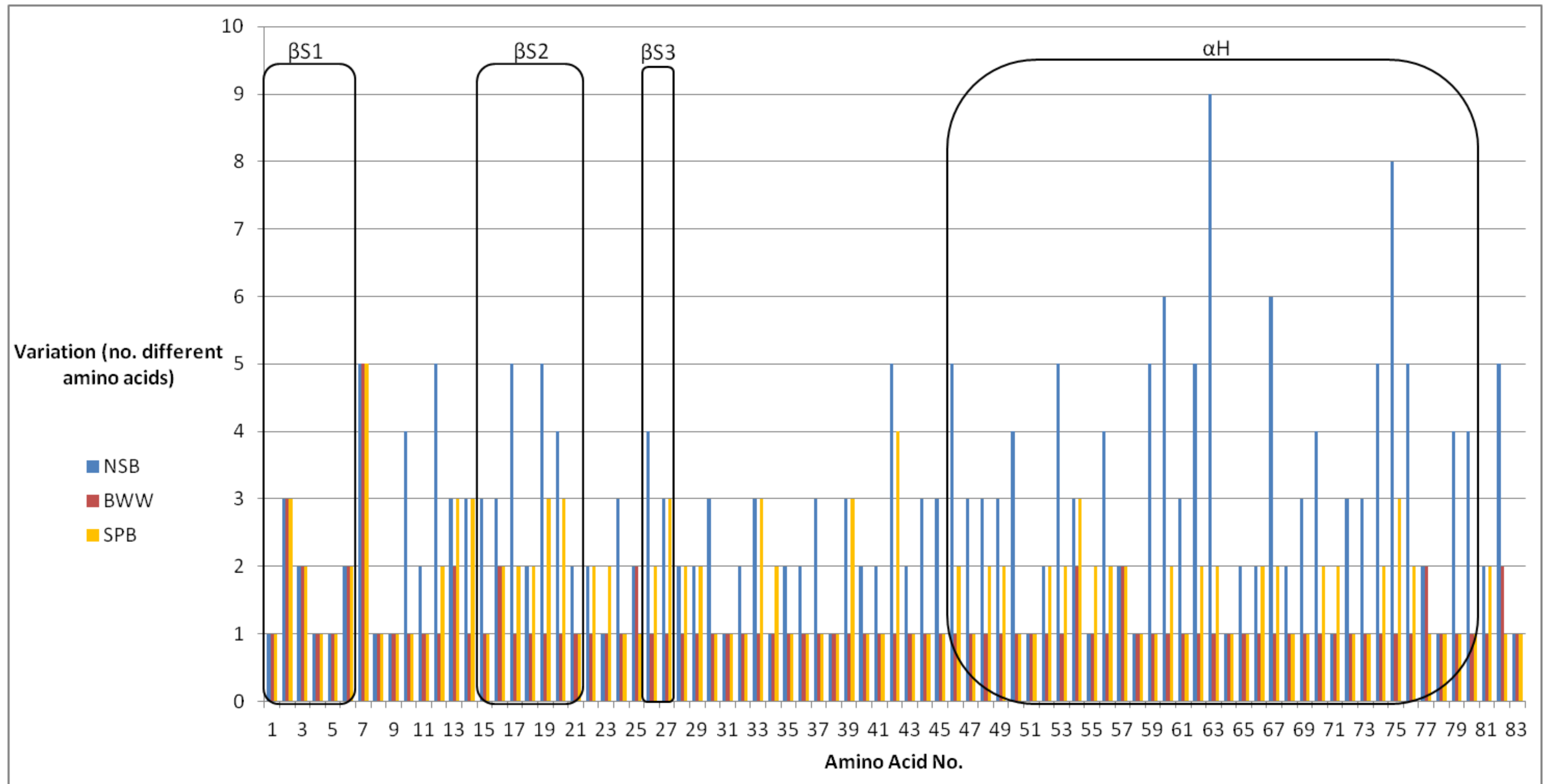


Figure 6.3 Histogram showing levels of variation for individual amino acids in the exon 2 region of the MHC Class II B in the NSB, BWW and SPB. Locations of β-sheet and α-helix domains are indicated by black boxes (βS - β-sheet; αH - α-helix) and were assigned based on She *et al.* (1991) and Miller & Lambert, (2004a).

compared to the NSB equivalent (Figure 6.3). Like the NSB, SPB sequences had the highest mean variation within the βS3 domain (2.50 amino acids per site), whereas the BWW βS1 domain had the highest observed variation (1.67), which is actually the lowest variation for any region (domain or inter-domain) in the NSB. And while the NSB and SPB both showed most of the amino acid variation within the β-sheet and α-helix domain regions (apart from βS1), the BWW sequences showed most of the highest variation in the inter-domain regions. However, nucleotide variation within the BWW gene was generally low with mean domain and inter-domain mean variation between 1.67 and 1 (cf 3.50 and 1.67 for NSB and 2.50 and 1.33 in SPB).

6.4 Discussion

Analysis of the diversity of the MHC Class II B in the BWW and SPB has revealed high nucleotide diversity for the PBR on the exon 2 in both species, matched the predictions based on what is known about exon 2 nucleotide diversity in the NSB (and the apparent low prevalence of pathogens in this species) and the relatively higher prevalence of pathogens observed for the BWW and SPB. In addition, the much lower genetic diversity observed for the exon 3 region also supported our hypothesis, since this region is generally more highly conserved than the highly polymorphic PBR. However, the level of diversity could not be fully evaluated for the PBR in the SPB as, in a similar scenario to the NSB, the sample size used appears to have been insufficient to capture all of the extensive variation observed in this species (Figure 6.2). This makes it difficult to draw direct inter-species comparisons but the overall high diversity (compared to other studies of passerine bird species) in all three species is in itself significant and allows us to speculate on what mechanisms may be driving such high levels of variation. However, there can be a high degree of confidence that all the available PBR variation for the BWW was captured using the given sample size (Figure 6.1) and, whilst the level of diversity is much higher than seen in many other passerine species (e.g. house sparrow; great reed warbler), it is lower than the common yellowthroat and collared flycatcher as well as both the SPB and NSB.

The higher prevalence of pathogens observed in the SPB and BWW (in comparison to the low prevalence recorded in NSBs) was hypothesised as being a strong driver of the evolution of MHC diversity in these species. *C. psittaci* may be endemic in populations in these species, with a 32% prevalence in BWWs and 24% in SPBs.

Although this is much lower than that observed in tits (Paridae) (Holzinger-Umlauf *et al.*, 1997), other studies of Chlymidiaceae in passerines have found very low incidences (Dovc *et al.*, 2005; Zwifel *et al.*, 2009). Pathogen-mediated evolution of the MHC Class II is understood to be an important factor in the unusual diversity of this gene group through balancing selection and this has been shown in passerines (e.g. Hawley & Fleischer, 2012). Given the high diversity of the exon 2 region in both the BWW and the SPB, combined with the high prevalence of *C. psittaci* in both species, it was expected that evidence of balancing selection would indicate pathogen-mediated evolution. However, the ratio of d_N vs. d_S for the exon 2 region in both species was low (Table 6.2) and was did not obviously differ from the d_N: d_S for the exon 3, for which lower values would be expected. Values for *p* were above the 0.05 significant level indicating these results were not significantly different from 1.
This suggests that balancing selection is not primarily responsible for the observed patterns of diversity in these species and the role of gene conversion or behavioural or demographic mechanisms may be important in the evolution of the MHC in these species. For example, Spurgin *et al.*, (2011) found that gene conversion in the Berthelot's pipit was responsible for the evolution of a large number of new MHC Class I haplotypes after a previous bottleneck reduced diversity in this gene group. Furthermore, Miller & Lambert (2004b) found that gene conversion was an important factor in the evolution of the MHC Class II B in two bird species of the genus *Petroica*. This theory is backed up by strong evidence of widespread gene conversion in the BWW, with 149 putative conversions identified in the 206 PBR sequences analysed. Therefore, it appears that gene conversion may be a more important factor driving MHC evolution in this species. However, there appears to be little evidence of gene conversion in the SPB PBR sequences, with just two events postulated. This is an important difference and underlines just how variable and complex MHC evolution is. So, while gene conversion appears to have generated significant levels of PBR diversity in the BWW, regardless of the presence of a potentially important pathogen such as *C. psittaci*, this does not appear to be the driver for the SPB.
Therefore, doubt still remains over the origin of MHC diversity in the SPB. Potentially the observed diversity is due to ancestral polymorphisms, such as has been suggested in *Neospiza* buntings (Jansen van Rensburg *et al.*, 2012). This would explain why there is no apparent evidence of balancing selection and gene

conversion. However, there is also the possibility that behavioural mechanisms may be responsible for generating the high levels of MHC diversity observed in the SPB. Little is known of the breeding behaviour of the SPB but since on Barrow Island they are mainly seen in pairs or family groups (Wooller & Bradley, 1981; Ambrose & Murphy, 1994) it could possibly be assumed that they are socially monogamous. However, social monogamy in other ecologically similar genera (e.g. fairy-wrens (Maluridae)) may commonly be supplemented by extra-pair copulations (EPC) (Mulder *et al.*, 1994). Although EPCs are thought to be rare in island populations (Griffith, 2000), it is a mechanism that has been shown to enhance fitness and genetic diversity in offspring in birds (Johnsen *et al.*, 2000; Foerster *et al.*, 2003). Furthermore, sexual selection has shown to have an equally important role in generating MHC diversity in some species (Penn & Potts, 1999; Penn 2002b). Therefore, breeding behaviour that facilitates EPCs may help to drive increased diversity in the MHC in SPBs. Nevertheless, without any evidence of polygamous breeding behaviour in this species, any strong conclusions are unable to be drawn and for now the explanation of ancestral diversity retained in the modern Barrow Island population appears to be the most likely.

By contrast, there is strong evidence for both balancing selection and gene conversion for the NSB PBR. As discussed in Chapter 5, the d_N:d_S ratio for the PBR sequences provides robust evidence for balancing selection, which was postulated as being driven by e.g. an unknown pathogen. However, no obvious candidate pathogens have been found in NSB pathology (Edmonds, 2007 unpublished). There was evidence of 254 gene conversion events from 260 PBR sequences and, although it is possible that some of these relate to PCR artefacts, by subscribing to similar protocols to those set out by Burri *et al*. (2014) it is expected that few of these events are false-positives. This allows the suggestion that, regardless of the presence or absence of a pathogenic driver, gene conversion alone may not be sufficient to explain the extremely high diversity observed for this region of the MHC. It is known that gene conversion rates can outstrip the rate of point mutations (Parham & Ohta 1996) by up to an order of magnitude (Spurgin *et al*., 2011) and this may explain why such a wealth of PBR diversity is found in an ostensibly genetically depauperate species. Nevertheless, given the strong evidence of balancing selection and the fact that the levels of PBR diversity are so much higher than would be

expected in the NSB, there remains the possibility that a driver of selection remains to be uncovered, be it a cryptic pathogen or an unknown behavioural mechanism.

From a conservation perspective, these results seem to be quite positive, since it appears that the NSB has not only managed to retain a vastly diverse MHC Class II B, which may improve its resilience to future pathogenic insults, but it also appears to have a mechanism of generating diversity, whether through selection or gene conversion or (more likely) a combination of these two mechanisms.

Nevertheless, without fully understanding the underlying mechanisms generating the high PBR diversity observed in this study, this study advocates only cautious optimism. While the NSB may have appeared to have dodged a genetic ‘bullet’ with the MHC Class II B, the same cannot necessarily be inferred for the rest of the genome. As previously discussed, inbreeding depression in small, bottlenecked populations can be a significant issue in the recovery of those populations and it remains crucial that genetic factors be considered in future management decisions of any threatened species.

Chapter 7 – Discussion

7.1 Microsatellite diversity and inbreeding in the noisy scrub-bird

To evaluate the extant levels of global genetic diversity in the endangered NSB, neutral markers were identified using second-generation sequencing and further characterised for levels of polymorphisms. Short-tandem repeats such as microsatellites can provide an insight into global diversity of the genome of a species (Hedrick, 1999; Hedrick, 2001) and may also be used to infer inbreeding in a population (Bruford & Wayne, 1993). However, these markers have been shown to be able to also produce misleading results, so should be treated with some caution and interpreted within a clear contextual understanding (Hedrick, 2001; Väli *et al.*, 2008).

Diversity in ten NSB-specific microsatellites was analysed across three subpopulations that were treated as discrete genetic populations (demes): the original parent population centred on Mt Gardner at Two Peoples Bay Nature Reserve (MG); the large translocated population from Angove Water Reserve to Arpenteur NR, centred on Mt Manypeaks (MP) and the translocated and genetically isolated population on Bald Island NR (BA). As predicted, allelic richness was generally low and comparable to other bird species known to have undergone significant population declines or bottlenecks (e.g. He *et al.,* 2006; Taylor & Jamieson, 2008; Chan *et al.,* 2011; Salmona *et al.*, 2012). The MG population showed marginally higher genetic diversity than both MP and BA and was the only deme found to possess private alleles. The MG and MP populations both deviated significantly from Hardy-Weinberg Equilibrium (HWE) and inbreeding coefficient (F_{IS}) values indicated high levels of inbreeding for both populations, although less so for MP. Linkage disequilibrium between up to three pairs of loci (MP only; MG and BA one pair each) provided more support for the hypothesis that bottlenecks/founder events had occurred in these populations. The BA population did not deviate significantly from HWE, with only one of ten loci showing any significant heterozygote deficiency and four loci actually showed heterozygote excess, although this can be symptomatic of a recent bottleneck (Cornuet & Luikart, 1996), which we understand to be the case. Heterozygote excess can arise through a process of binomial-sampling error (Pudovkin *et al.*, 1996; Luikart & Cornuet, 1999; Balloux, 2004; Allendorf &

Luikart, 2007) and may relate to the sampling effect that is imposed through the randomly selection of individuals for translocation.

Structural analysis found little evidence of genetic clustering between postulated demes, although there was evidence for clustering within the MG and MP populations. Nevertheless, there was evidence for significant genic differentiation (F_{ST} & Fisher's Exact test) between the MP and BA demes, presumably resulting from sampling for translocations.

The effective size for the foundation population for BA was estimated and, although the results were close to what was predicted (nine from 11 birds released), the 95% confidence interval was too broad to allow any strong conclusions. A similar result was also obtained for the estimate of N_e with the result of 42 having a 95% confidence interval of 22 to 184, although even the upper bound of these estimates is still relatively low given the population index of 133-166 territorial males over the sample period. This potentially lends additional weight to the theory that this species is at least somewhat polygynous. Additionally, an attempt to model the presence of bottlenecks in all three demes was similarly unsuccessful, mainly due to the low resolution provided by just ten loci, since the software used (BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996)) requires at least 20 loci to produce a robust result. However, knowledge of the history of these populations means it is possible to have a high confidence in the occurrence of bottlenecks.

While the sample sizes of 22, 23 and 15 from each population are relatively small, this is a result of several factors including the difficulties involved in capturing live NSBs (2.2.2) and the limited availability of pre-existing tissue samples of sufficient quantity, quality and age. However, modelling by Lowe *et al.* (2004) predicts that 20 individual samples from a population is sufficient to capture 80% of available variation, although this may be higher in populations with low genetic diversity, as is predicted for the NSB. In addition, Hale *et al.* (2012) found that sampling more than 25-30 individuals per population in a microsatellite diversity study was of limited value, and, for estimates of expected heterozygosity (H_E), 20 individuals was generally sufficient. However, while this study may be pertinent to a wide range of genetic diversity studies, these estimates may be different for other population genetics assessments, e.g. allelic richness, differentiation etc, as in the case of this study. Furthermore, the authors warn that sample sizes below 20 should be avoided. However, the 15 samples obtained from BA were the result of considerable

expenditure of time and resources and given the tiny founder group for this population and the fact the sample size for the other two demes is above the threshold of 20 samples, 15 samples from BA were deemed to be satisfactory. Furthermore, the allele discovery curves generated for all populations indicated that sample sizes were sufficient to reveal most of the available diversity at the loci being investigated.

In summary, these results generally matched the expectations of genetic diversity and inbreeding for a bottlenecked bird species, although more evidence of inbreeding in BA was predicted, which was founded with just 11 individuals. Indeed, this population is apparently not significantly inbred and the presence of heterozygote excess at four loci provides evidence that this may be due to binomial sampling error (caused by random selection of males and females for translocation), which has been sustained through strong population growth. Nevertheless, in all populations diversity was generally low, and the indication that MG and potentially MP populations are significantly inbred is of concern. However, the potential weakness of neutral variation as a predictor of overall fitness and evolutionary potential must be acknowledged and to draw more solid conclusions on the genetic diversity of the NSB, the level of variation at an adaptive marker was investigated.

7.2 MHC Class II B diversity in the noisy scrub-bird

A total of 383 sequences of the exon 2 and 222 sequences of the exon 3 region of the Major Histocompatibility Complex (MHC) Class II B of the NSB were obtained from 30 individual NSBs (ten from each postulated deme). This region of the MHC Class II B codes for the functionally important peptide-binding region (PBR). Genes coding for MHC proteins are the most polymorphic in vertebrates (Penn, 2002a) and the PBR as the functional part of the protein is a key component of this variation. Of the two exons, exon 3 is more conserved. 216 different sequences ('alleles') were obtained for exon 2 which appeared to be functional and this represents quite surprising diversity given the context of the species' population history and when compared to the MHC Class II B in other passerines (Westerdahl *et al.*, 2000; Bonneaud *et al.*, 2004; Miller & Lambert, 2004a; Aguilar *et al.*, 2006). However, the observed sequence variation was generally restricted to the regions encoding for the α-helix and the three β-sheet domains of the PBR and other regions were more

conserved. Furthermore, it appears that only a portion of the available PBR diversity in the NSB has been sequenced. However, similarly high diversity has been observed in the common yellowthroat (*Geothlypis trichas*) (Bollmer *et al.*, 2010) and collared flycatcher (*Ficedula hypoleuca*) (Zagalska-Neubauer *et al.*, 2010), so these results are not without precedent. What is surprising is that these results were not obtained from a widespread, outbred species but one with a highly restricted distribution and has undergone one or more bottlenecks relatively recently.

Pathogens are believed to be important drivers of MHC diversity through the process of balancing selection, and strong evidence was found that balancing selection has been responsible for generating diversity. The proportion of non-synonymous (d_N) base substitutions in the exon 2 sequences was significantly higher than for synonymous (d_S) substitutions. In contrast, the opposite was true for the exon 3 region, adding support to this theory in this species, since exon 2 contains more of the functional domains of the PBR. Furthermore, there is evidence from other studies of balancing selection maintaining MHC diversity through a population bottleneck (Hawley & Fleischer, 2012; Oliver & Piertney, 2012). However, there is little evidence of clinical disease in NSBs, or any high prevalence of parasites or other pathogens. Indeed, the incidence of blood parasites (malarial or filarial) was generally much lower than observed generally in other birds (Edmonds, 2007 unpublished). This represents a further paradox in this species. Having such high PBR sequence diversity may suggest a pathogen may have remained undiscovered e.g. filarial nematodes which are more apparent in samples taken at night than during the day (Reppas *et al.*, 1995). The theory of 'parasite release', provides one possible explanation, as introduced species may experience a reduction in pathogen diversity through a founder event (i.e. a bottleneck) (Torchin *et al.*, 2003). Furthermore, pathogens may be lost from a population that is subject to a contraction since hosts with lower fitness may be less likely to survive the bottleneck and the absence of hosts would lead to the extirpation of the pathogen from that population. Alternatively, the level of resistance conferred by the high diversity observed in the NSB may inhibit pathogens so they are essentially undetectable in the population. Finally, there are other mechanisms that are understood to possibly drive MHC evolution, such as sexual selection, that may be to some extent responsible for the extremely high exon 2 diversity observed. To better understand the possible reasons influencing this level of variation, the MHC study was repeated using two similar

passerine bird species, for which there is evidence for at least one highly prevalent pathogen.

7.3 MHC Class II B diversity in Barrow Island birds

The SPB and BWW from Barrow Island represent two species populations that have a relatively high incidence of *Chlamydophila psittaci* (23% in SPB; 32% in BWW), as well as other pathogens including coccidia. The exon 2 and 2 were sequenced for individuals from these populations. It was predicted that both species would show a higher sequence diversity of the PBR than for other passerines, including that of the NSB (although this was unlikely to be elucidated by the given sample size). In addition, it was predicted that this diversity would be driven by balancing selection, evidence of which would come from a higher proportion of d_N to d_S. As predicted, from 206 sequences from 15 BWWs 87 functional sequences were obtained and from 185 sequences from 14 SPB 110 functional sequences were obtained. Allele discovery curves for each species indicate that, while little BWW variation remains undiscovered, the level of diversity in the SPB is likely to be much higher than demonstrated here. By comparison, exon 3 sequence diversity was much lower, with 16 and 45 functional sequences for the BWW and SPB respectively. However, there was no strong evidence for balancing selection being the main driver of MHC diversity in either species. If the strain of *C. psittaci* occurring on Barrow Island has a low virulence, the pathogen may not exert a strong selection pressure, either through increased mortality or a reduction in fitness. Therefore, balancing selection will tend to be less important than other mechanisms in driving MHC evolution and the high diversity of the MHC Class II B in these species is more likely to be driven by other mechanisms, for example, sexual selection. Nevertheless, this discovery underlines the complexity of the evolution of the MHC.

Screening of BWW and NSB PBR sequences revealed evidence that gene conversion has been a key mechanism in the generation of diversity for this gene region. Gene conversion is believed to generate variation more rapidly than simple point-mutations, and has been shown to restore variation in bottlenecked species (such as the NSB). Therefore, this may help explain why such high levels of PBR diversity in the BWW and NSB have been observed. However, there was little evidence of gene conversion in the SPB and this, combined with a similar dearth of evidence for

balancing selection, suggests that the high level of PBR diversity in this species may either be due to ancestral polymorphisms that have been inherited and maintained, or to behavioural mechanisms, e.g. sexual selection.

Incorporation of the analysis of the diversity of the MHC Class II B on these Barrow Island bird species was done to aid the understanding of the processes that have generated and maintained the extraordinary levels of PBR sequence diversity in the NSB. It seems likely that both balancing selection and gene conversion have had a key role in this, the latter potentially crucial to the maintenance of such high diversity through at least one population bottleneck. However, it is still unclear what mechanism may be driving selection in this marker. With scant evidence of disease or parasites, there remain other possibilities. Firstly, as discussed in 7.2, there is the possibility of the presence of a cryptic pathogen that has not been identified as yet. Another possible process that could be driving balancing selection is sexual selection, i.e. through disassortative mating. For example, although the NSB was previously thought to be socially monogamous, it is now believed more likely to be polygynous to at least some extent (based on sexual dimorphism, breeding behaviour and social interactions between males). Therefore, in this case female choice becomes very important and if this includes MHC compatibility and heterozygosity, it could be a key driver of diversity in this gene group. Furthermore, since the female NSB only lays one egg, the investment in a single offspring increases the need for 'good' genes. The fact that the populations on Mt Manypeaks and Bald Island have experienced strong population growth, in spite of very small founder sizes, indicates that fitness is relatively high in these populations. If female mate choice is as important as is suggested in driving MHC evolution in this species, it may help to explain how high reproductive productivity (driven by high fecundity and offspring survivorship) has been maintained, in spite of low levels of neutral variation. Finally, the retention of similar levels of neutral and adaptive diversity (compared to the original source population) in the Bald Island population, which was established with just three females, may be due to mate choice by females who may have helped to maximise heterozygosity in the population by choosing genetically dissimilar mates . The mechanisms that might mediate and facilitate female mate choice in this way are unknown, as there is no evidence of e.g. olfactory cues such as those recorded in blue petrels (Strandh *et al.*, 2012). However, other physical cues could potentially be important such as morphological symmetry (which has been found to be an indicator

of MHC (HLA) heterozygosity in humans (Roberts *et al.*, 2005)) or the powerful calling of territorial males, although correlation of 'good' genes and the vocal ability of an individual currently lacks empirical evidence in this species. However, the possibility that females are helping to drive the evolution of MHC diversity in this species is both intriguing and tantalising. In conclusion, the levels of diversity in the MHC Class II in the NSB are unprecedented in the context of this study and the results from Barrow Island tend to muddy the waters rather than elucidate possible reasons for this observation. There are various mechanisms that might be driving this diversity, including gene conversion which is implicated in driving MHC evolution in the NSB. However, theories involving behavioural mechanisms are somewhat conjectural and further work is required to gain a better understanding what is driving this diversity and its significance for the ongoing management of the NSB.

7.4 Implications for conservation management

The primary aim of this study was to help inform future management for the continued conservation of the NSB by providing a better understanding of the population genetics of the species. One caveat with these results is the temporal variation between the study populations, with MG being sampled in between 1998 and 2001, MP between 2000 and 2007 and BA between 2009 and 2011. These temporal as well as spatial differences need to be accounted for, as the genetic scenario of a population over 10 years ago may be different to the present situation. Microsatellite work in this study has suggested that levels of neutral variation are low, but this cannot reasonably be extrapolated to cover levels of adaptive variation, since this study has found that levels of diversity in the important immunological gene group, the MHC Class II B, are unexpectedly high across all populations. However, the microsatellite data has uncovered evidence that inbreeding levels are high in the parent population (MG) and potentially significant in MP. Despite high levels of MHC diversity, inbreeding depression may still impact these populations as other genes may conform to the patterns of diversity shown by the microsatellites. Inbreeding depression may have a significantly detrimental effect on a population, either slowly through a loss of adaptive potential caused by a loss of genetic diversity or more rapidly through the increased probability of recombination of deleterious recessive alleles (Keller & Waller, 2002). The occurrence of a nestling with

presumed 'rubber-bill' syndrome (Tiller *et al.*, 2007) and an adult with only one wing (A. Danks *pers. comm.*) on Mt Gardner has already created some concern and provided the initial impetus for this study. With a cryptic and rarely observed species such as the NSB, regular surveys are essential to monitor population levels and recognise any signs of a possible unexplained decline. However, by the time a decline is identified it may actually be too late to intervene. This study advocates an effort to alleviate the potential for inbreeding depression by encouraging gene flow between existing populations, through either translocation (i.e. assisted migration or genetic rescue) or by increasing habitat connectivity, e.g. between Mt Gardner and Angove Water Reserve. Movement of birds between MG and MP and the BA deme would also be prudent given the significant differentiation between MP and BA. However, even though it has slightly lower allelic diversity than both MG and MP, BA holds the highest proportion of heterozygous individuals of the three populations. Therefore, any one individual is likely to have more genetic variability than any individual from either of the other populations. The striking paradox, therefore, is that the population founded with just 11 individuals now theoretically represents the best source of birds for genetic rescue, or indeed translocation to establish new populations.

When the locations where animals were captured for the translocations to Mt Manypeaks and Bald Island are compared, approximately 45% (n = 14) of animals used in the Mt Manypeaks translocation were sourced within an area smaller than $0.25km^2$ in the Wilson's Swamp/Gardner Creek area of TPB, while the 11 animals used for translocation to Bald Island came from an area larger than $5.5km^2$. Therefore, while fewer animals were released on Bald Island than on Mt Manypeaks, a large proportion of the Mt Manypeaks birds were captured from a small geographic area. Assuming that many of these birds are somewhat related, this has potentially increased the risk of subsequent inbreeding in this population. This may be an important causal factor in the observed levels of heterozygosity and genic differentiation in these two populations and future translocations should seek to sample individuals from as broad a geographic area as possible.

Translocations of birds to new areas have been a key component of conservation management for the NSB (Comer *et al.*, 2010) and the foundation of new populations elsewhere should remain an important safeguard against stochastic events (e.g.

wildfire; disease) that may potentially wipe out an entire population. However, in the short-term it may be a more judicious use of resources to concentrate on supplementing genetic diversity in existing populations. This is already being undertaken, with translocations from Bald Island being used to re-establish a population within the Angove Water Reserve in 2011 and 2012, which over time could also improve gene flow between MP and MG. Once successfully established, future translocations from other populations may help augment the genetic variability in this new population. Pacioni *et al*. (2013) produced a list of recommendations for future translocations of woylies (*Bettongia pencillata*) but many of their points are also pertinent for NSB management, for example, the need for a large founder group (>50) (also supported by Hale & Briskie (2004) and Tracy *et al*. (2011)), choosing founders to maximise genetic variability and the selection of introduction sites with high connectivity to other suitable locations.

Fortunately, many of these recommendations are already being implemented in the current management strategies for the NSB. However, the resources required to capture and translocate more than 50 NSBs would be considerable, given the highly elusive nature of this species. Post-release monitoring is also challenging and generally restricted to short-term radio-tracking (S. Comer *pers. comm.*). Finally, another recommendation of Pacioni *et al.* (2013) is for the modelling of a population viability analysis (PVA) to inform management options, which is again difficult in such a cryptic species as little is known of its the biology and breeding ecology. A PVA model performed for the Bald Island population indicated that the population should have become extinct some time ago (S. Comer, *pers. comm.*). This is clearly not the case, with 153 territories recorded in 2014. Microsatellite data was also used to estimate N_e in the putative MG deme and although the 95% confidence interval was very broad, the estimates were all lower than would be predicted if the reproductive sex ratio was 1:1. This lends additional weight to the theory that NSBs are not socially monogamous but polygynous. From a conservation perspective, low N_e will make it harder for populations to recover and will compound genetic diversity loss (Stiver *et al*., 2008).

The disclosure of substantial levels of diversity of the PBR of the MHC Class II B was in pleasant contrast to the levels of microsatellite diversity. It is hoped that, regardless of the mechanism driving this diversity, this would help the resilience of

the species to exposure to a new pathogen. Certainly, there is evidence of rapid evolution in this gene, although as to why the NSB has such high MHC diversity remains a mystery. Therefore, given that the mechanisms behind and the implications of the observed levels of variability are not fully understood, caution should be taken in the interpretation of these results. In the event that a new-and-emerging disease became a potential threat, it is safest to presume that the NSB will be vulnerable to it. The strong evidence for balancing selection in the PBR indicates the presence of one or more mechanisms driving selection in this species. While the existence of a cryptic pathogen cannot be excluded, the possibility remains that it may be driven by sexual selection in the form of disassortative mating. Given the weight of evidence for polygyny in this species (versus the traditional belief of social monogamy) it seems possible that inter-gender competition in males and mate choice in females may be important factors in the mating system of the NSB. This has implications for a) N_e as polygamous mating systems will reduce the effective number of breeders (Nunney, 1993) and b) sex ratios in natural populations, which may be skewed and therefore may influence the effectiveness of translocations. However, the latter is confounded by the sex ratio of 8:3 in the successful establishment of the Bald Island population, since it might be expected for a higher ratio of females to males in polygynous mating system to be more appropriate.

7.5 Future directions

Given the cryptic nature of the NSB, any future studies to further knowledge of this elusive species will entail a great deal of effort to acquire a sufficiently robust dataset, regardless of the nature of the study. The best way to build on what is reported here would involve the construction of pedigrees, which would entail the capture and genotyping of a large number of birds from a given location. This would require a huge input of time, effort and resources. Therefore, future directions in genetics may be better focussed on data already gathered in the course of this study, for example, the second-generation sequencing (SGS) data used to identify and characterise the 10 microsatellite loci. A BLAST search with the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) with the SGS data provided hits for MHC Class I and II as well as mitochondrial control regions, and there is scope to data-mine even more. Despite this, from a conservation perspective, the value in this

would be minimal, except perhaps examining diversity in the MHC Class I gene group for a comparison with the MHC Class II. However, the sequencing of the whole NSB genome would provide further opportunities for examining genetic in a range of other markers (outlined in Steiner *et al.*, 2013) with the advent of second- and third-generation sequencing technology, this goal may be realistically attainable.

However, there may be value in the identification and analysis of similar numbers of microsatellite loci in the SPB and BWW, to complete the comparison between the NSB and these species with a comparison of neutral vs. adaptive variability in the Barrow Island populations of these species. Given the huge dichotomy between variation at neutral and adaptive markers in the NSB, it would be interesting to see if results for the Barrow Island birds were comparable, given the expectations of low diversity in island populations (Frankham, 1997; Frankham, 1998).

In addition to microsatellites, there could arguably be some value in undertaking single-nucleotide polymorphism (SNP) discovery and analysis. This technique has been used in population genetics studies (e.g. grey wolf (*Canis lupus*) (Seddon *et al.*, 2005; brown bear (*Ursus arctos*) (Fahlén, 2014)) and Morin *et al.* (2004) review the other possible uses of SNPs in conservation genetics, including genetic diversity, N_e and population structure. A study of the banded wren (*Thryothorus pleurostictus*) used 90 anonymous loci to identify 168 SNPs and used these markers to undertake analyses like deviation from HWE, LD and sex linkage (Cramer *et al.*, 2008). The study concluded that in species with low microsatellite diversity (e.g. the NSB), SNPs could present an excellent practical alternative. Morin *et al.* (2009) stated that for statistically powerful analyses, a sample of 100 individuals per population with >75 SNPs would be necessary, although allowances could be made for smaller samples when. However, in light of the NSB's low microsatellite diversity we might expect that considerably more SNPs would be necessary to draw robust conclusions and the value of this information must outweigh the resources required to obtain it. Nevertheless, if resources were available, a SNP-based study of the NSB may help elucidate some of the paradoxes uncovered by this study.

An alternative to the use of the MHC is another group of immunological genes that exhibit a relationship between variation and disease resistance/susceptibility known as Toll-like receptors (TLRs). TLRs have a key role in initiating innate immune response and genetic studies have found relationships between variation in these markers and disease susceptibility (e.g. Schröder & Schumann, 2005; Netea *et al.*,

2012). However, TLRs have only recently been used in conservation genetics studies, for example an examination of genetic diversity in 10 threatened birds from New Zealand which provided evidence of genetic drift shaping post-bottleneck TLR diversity in these species, as well as providing data which could be compared with more widespread species (Grueber *et al*., 2015). It appears that TLRs could represent an important alternative to MHC markers in conservation genetics and while the MHC is still of major utility, the high levels of diversity observed in this gene group can prove problematic for obtaining genotypes, in contrast to TLR genotypes, which are much simpler to obtain (Grueber *et al*., 2012). Therefore, a comparison of MHC and TLR diversity would be an interesting development of this work.

Another direction in genetics that is yet to be taken is examining historical diversity of both the MHC and microsatellites in the NSB. This was beyond the remit of this study but presents some interesting questions, for example: how does contemporary diversity differ from that when the species was still widespread throughout south-west Western Australia? However, working with ancient DNA is a highly specialist field and this type of study would require collaboration with experts to ensure successful extraction and amplification from historical specimens.

As previously discussed, the driving factor(s) behind the extraordinary levels of MHC diversity in the NSB are currently unknown, although it is hypothesised that a cryptic pathogen or disassortative mating are the most likely causes. However, to exclude the possibility of a cryptic pathogen and assess the prevalence of pathogens in the NSB's environment, it would be valuable to undertake health screening of other bird species that co-exist with the NSB. A banding study that incorporated health-screening at a location such as Two Peoples Bay Nature Reserve would be an ideal way to ascertain the background prevalence of avian pathogens, including elements that were not picked up in NSBs such as malarial and filarial blood parasites.

This study will hopefully help to inform future management of the NSB, but to derive the greatest benefit from this work, the best outcome were if some or all of these directions were explored in the near-future.

Appendices

Appendix i - List of noisy scrub-bird samples

Bird ID	Capture Subpopulation	Year	Sex	Tissue Type
98M02*	MG	1998	M	Blood
98M04	MG	1998	M	Blood
98M05	MG	1998	M	Blood
98M06	MG	1998	M	Blood
98M07*	MG	1998	M	Blood
98M08	MG	1998	M	Blood
98M09*	MG	1998	M	Blood
98M10	MG	1998	M	Blood
98M11*	MG	1998	M	Blood
98M12*	MG	1998	M	Blood
98M13	MG	1998	M	Blood
98M14	MG	1998	M	Blood
99F01	MG	1999	F	Blood
99F03*	MG	1999	F	Blood
99M05*	MG	1999	M	Blood
99M06*	MG	1999	M	Blood
99M07	MG	1999	M	Blood
99M08	MG	1999	M	Blood
99M15*	MG	1999	M	Blood
00M01*	MP	2000	M	Blood
00M02*	MP	2000	M	Blood
00M03	MP	2000	M	Blood
00M04	MP	2000	M	Blood
00M08*	MP	2000	M	Blood
00M09	MP	2000	M	Blood
00M11	MP	2000	M	Blood
00M12	MG	2000	M	Blood
00M13	MG	2000	M	Blood

Bird ID	Capture Subpopulation	Year	Sex	Tissue Type
01F01*	MP	2001	F	Blood
01M01*	MP	2001	M	Blood
01M02*	MP	2001	M	Blood
01M04*	MG	2001	M	Blood
01M05*	MP	2001	M	Blood
01M07	MP	2001	M	Blood
01M09	MP	2001	M	Blood
01M10	MP	2001	M	Blood
01M11	MP	2001	M	Blood
01M12	MP	2001	M	Blood
01M13	MP	2001	M	Blood
06M03*	MP	2006	M	Blood
06M05*	MP	2006	M	Blood
06M07	MP	2006	M	Blood
07M03	MP	2007	M	Blood
07M04	MP	2007	M	Blood
07M05*	MP	2007	M	Blood
09M01	BA	2009	M	Feathers
09M02*	BA	2009	M	Blood & Feathers
09M03	BA	2009	M	Feathers
10F01*	BA	2010	F	Blood & Feathers
10M01	BA	2010	M	Blood & Feathers
10M02*	BA	2010	M	Blood & Feathers
10M03*	BA	2010	M	Blood & Feathers
10M04	BA	2010	M	Blood & Feathers
10M05*	BA	2010	M	Blood & Feathers
11M01*	BA	2011	M	Blood & Feathers
11M02*	BA	2011	M	Blood & Feathers
11M03*	BA	2011	M	Blood & Feathers
11M04	BA	2011	M	Blood & Feathers
11M05*	BA	2011	M	Blood & Feathers

Bird ID	Capture Subpopulation	Year	Sex	Tissue Type
11M06*	**BA**	**2011**	**M**	**Blood & Feathers**

***, individuals used for MHC sequencing; MG, Mt Gardner and Lakes Gardner and Moates (Two Peoples Bay Nature Reserve); MP, Mt Manypeaks Nature Reserve, Angove Water Reserve (Watercorp), Waychinicup National Park and Arpenteur Nature Reserve; BA, Bald Island Nature Reserve (all DPaW tenure or Unallocated Crown Land unless stated); M, Male; F, Female**

Appendix ii - List of black-and-white fairy-wren & spinifexbird samples

Spinifexbird samples

Code	Year	Sex	Tissue Sampled
SPB 10/01	2010	Male	Blood Feathers
SPB 10/02*	2010	Male	Blood Feathers
SPB 10/03	2010	Male	Blood Feathers
SPB 10/04	2010	Male	Blood Feathers
SPB 10/06	2010	Male	Blood Feathers
SPB 10/07	2010	Female	Blood Feathers
SPB 10/08	2010	Male	Blood Feathers
SPB 10/10	2010	Male	Blood Feathers
SPB 10/11	2010	Male	Blood Feathers
SPB 10/12	2010	Female	Blood Feathers
SPB 10/13*	2010	Female	Blood Feathers
SPB 10/14	2010	Male	Blood Feathers
SPB 10/15	2010	Male	Blood Feathers
SPB 10/16	2010	Male	Blood Feathers
SPB 10/17	2010	Female	Blood Feathers
SPB 10/18	2010	Female	Blood Feathers
SPB 10/19	2010	Male	Blood Feathers
SPB 10/20	2010	Male	Blood Feathers
SPB 10/21	2010	Male	Blood Feathers
SPB 10/23	2010	Male	Blood Feathers
SPB 10/24	2010	Male	Blood Feathers
SPB 10/25	2010	Male	Blood Feathers
SPB 10/26	2010	Male	Blood Feathers
SPB 10/27	2010	Male	Blood Feathers
SPB 10/29	2010	Female	Blood Feathers
SPB 10/30	2010	Male	Blood Feathers
SPB 10/31	2010	Male	Blood Feathers
SPB 10/32	2010	Male	Blood Feathers

Code	Year	Sex	Tissue Sampled
SPB 10/33	**2010**	**Male**	**Blood Feathers**
SPB 10/34	**2010**	**Male**	**Blood Feathers**
SPB 10/35	**2010**	**Male**	**Blood Feathers**
SPB 10/36	**2010**	**Male**	**Blood Feathers**
SPB 10/37	**2010**	**Male**	**Blood Feathers**
SPB 10/38	**2010**	**Male**	**Blood Feathers**
SPB 11/01*	**2011**	**Male**	**Blood Feathers**
SPB 11/02*	**2011**	**Male**	**Blood Feathers**
SPB 11/03*	**2011**	**Male**	**Blood Feathers**
SPB 11/04	**2011**	**Male**	**Blood Feathers**
SPB 11/05*	**2011**	**Female**	**Blood Feathers**
SPB 11/06*	**2011**	**Male**	**Blood Feathers**
SPB 11/07*	**2011**	**Male**	**Blood Feathers**
SPB 11/08*	**2011**	**Female**	**Blood Feathers**
SPB 11/09*	**2011**	**Male**	**Blood Feathers**
SPB 11/10*	**2011**	**Male**	**Blood Feathers**
SPB 11/11	**2011**	**?**	**Blood Feathers**
SPB 11/12*	**2011**	**Male**	**Blood Feathers**
SPB 11/14*	**2011**	**Male**	**Blood Feathers**
SPB 11/15*	**2011**	**Male**	**Blood Feathers**
SPB 11/16	**2011**	**Male**	**Blood Feathers**

(* samples used for sequencing of MHC Class II)

Black-and-white fairy-wren samples

Code	Year	Sex	Tissue Sampled
BWW 10/02*	**2010**	**Male**	**Blood Feathers**
BWW 10/03	**2010**	**?**	**Blood Feathers**
BWW 10/04	**2010**	**Female**	**Blood Feathers**
BWW 10/06	**2010**	**Female**	**Blood Feathers**
BWW 10/08*	**2010**	**?**	**Blood Feathers**
BWW 10/10*	**2010**	**Male**	**Blood Feathers**

Code	Year	Sex	Tissue Sampled
BWW 10/11	**2010**	**Male**	**Blood Feathers**
BWW 10/12	**2010**	**?**	**Blood Feathers**
BWW 10/13	**2010**	**Male**	**Blood Feathers**
BWW 10/14*	**2010**	**Female**	**Blood Feathers**
BWW 10/15*	**2010**	**Female**	**Blood Feathers**
BWW 10/16	**2010**	**Male**	**Blood Feathers**
BWW 10/17*	**2010**	**Female**	**Blood Feathers**
BWW 10/18	**2010**	**Female**	**Blood Feathers**
BWW 10/19	**2010**	**Female**	**Blood Feathers**
BWW 10/20	**2010**	**Male**	**Blood Feathers**
BWW 10/21	**2010**	**Male**	**Blood Feathers**
BWW 10/22	**2010**	**Male**	**Blood Feathers**
BWW 10/23*	**2010**	**Female**	**Blood Feathers**
BWW 10/24	**2010**	**Female**	**Blood Feathers**
BWW 10/26*	**2010**	**Male**	**Blood Feathers**
BWW 10/28	**2010**	**Female**	**Blood Feathers**
BWW 10/29	**2010**	**Male**	**Blood Feathers**
BWW 10/30	**2010**	**Female**	**Blood Feathers**
BWW 10/31*	**2010**	**Male**	**Blood Feathers**
BWW 11/01	**2011**	**Male**	**Blood Feathers**
BWW 11/02*	**2011**	**Female**	**Blood Feathers**
BWW 11/03*	**2011**	**Male**	**Blood Feathers**
BWW 11/04*	**2011**	**Female**	**Blood Feathers**
BWW 11/06*	**2011**	**Female**	**Blood Feathers**
BWW 11/07*	**2011**	**Female**	**Blood Feathers**
BWW 11/09	**2011**	**Male**	**Blood Feathers**
BWW 11/10	**2011**	**?**	**Blood Feathers**
BWW 11/12*	**2011**	**Male**	**Blood Feathers**
BWW 11/15	**2011**	**Female**	**Blood Feathers**

(* samples used for sequencing of MHC Class II)

Appendix iii Summary of pathology results for black-and-white fairy-wrens (BWW) and spinifexbirds (SPB) captured for translocation from Barrow Island to Hermite Island in 2010

ID	Protozoa/Cyst/Ova	Gram Ratio	Culture	Chlamydophila
BWW01	-	>10:1	-	NT
BWW02	Coccidia +	>10:1	Enterobacter +++	-
BWW03	-	>10:1	-	NT
BWW04	-	>10:1	Enterobacter +++	-
BWW05	-	1:1	Enterobacter +++	-
BWW06	-	>10:1	Non Haem E coli +++	-
BWW08	-	>10:1	-	-
BWW09	-	>10:1	-	-
BWW11	-			-
BWW12	-	>10:1	-	NT
BWW16	-	>10:1	-	-
BWW17	-	10:1	Enterobacter +++	-
BWW18	-	1:1	Enterobacter +	-
BWW19	-	>10:1	Non Haem E coli ++	-
BWW20	-	>10:1	Enterobacter & P.aeruginosa ++	-
BWW21	-		-	+
BWW22	-	>10:1	-	-
BWW23	-	>10:1	Enterobacter ++	+
BWW24	-	>10:1	Enterobacter +	+
BWW26	-	>10:1	-	+
BWW28	-		-	+
BWW29				+
BWW31	-	>10:1	-	NT
SPB01	-	>10:1	Non Haem E.coli +	-
SPB02	-	>10:1	Enterobacter ++	-
SPB03	-	5:1	Proteus +++	-
SPB04	-	2:1	Pseudomonas aeruginosa +++	-
SPB05	-	1:1	-	-
SPB07	-	>10:1	-	-
SPB08	Coccidia +	>10:1	Non Haem E.coli +++	-
SPB10	-	1:2	Enterobacter +++	-
SPB11	-	>10:1	-	NT
SPB12	Coccidia +	1:1	Enterobacter A & B ++	-
SPB13	-	>10:1	Enterobacter +	+

ID	Protozoa/Cyst/Ova	Gram Ratio	Culture	Chlamydophila
SPB14	-	5:1	Enterobacter ++	-
SPB15	-	1:1	Enterobacter +++	+
SPB16	-	>10:1	-	-
SPB17	-	>10:1	Non Haem E coli +	-
SPB18	-	>10:1	-	+
SPB19	-	>10:1	Enterobacter ++	-
SPB20	-	>10:1	Enterobacter +	-
SPB21	-	1:5	Enterobacter +++	-
SPB??	Coccidia +	3:1	Enterobacter +++	-
SPB23	-	1:10	Enterobacter +	-
SPB24	-	>10:1	-	NT
SPB25	-	>10:1	Enterobacter +	+
SPB26	-	1:10	Enterobacter +++	-
SPB27	-	5:1	Enterobacter ++	-
SPB??	-	>10:1	-	+
SPB28	-	1:1	Enterobacter ++	-
SPB29	-	>10:1	-	-
SPB30	-	1:1	Enterobacter ++	+
SPB31	-		-	-
SPB32	-		-	-
SPB33	-		-	+
SPB35	-		Non Haem E coli +	-
SPB36	-	>10:1	-	-
SPB37	-	>10:1	-	+
SPB38	-	>10:1	-	-

+ light growth; ++ moderate growth; +++heavy growth; - negative; NT not-tested; gram ratio is gram positive: gram negative (positive usually > negative) (data courtesy of DPaW and Vetpath 2010).

Appendix iv - Qiagen DNeasy Blood and Tissue Kit Protocol: Purification of Total DNA from Animal Blood (Spin-Column Protocol) (adapted for nucleated erythrocytes) (Qiagen, 2006)

Important points before starting

All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.

Vortexing should be performed by pulse-vortexing for 5–10 s.

Things to do before starting

Buffer AL may form a precipitate upon storage. If necessary, warm to 56°C until the precipitate has fully dissolved.

Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution. Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.

Procedure

1. Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube (not provided). Add 5–10 µl anticoagulated blood. Adjust the volume to 220 µl with PBS. Continue with step 2.

Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml) and incubate for 2 min at room temperature before continuing with step 2.

2. Add 200 µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.

Ensure that ethanol has not been added to Buffer AL (see “Buffer AL”, page 18). Buffer AL can be purchased separately (see page 56 for ordering information). It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

3. Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.

It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

4. Pipet the mixture from step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.*

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane.

Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.

Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. Recommended: For maximum DNA yield, repeat elution once as described in step 7.

This step leads to increased overall DNA yield. A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Appendix v - QIAquick Gel Extraction Kit Protocol (using a microcentrifuge) (Qiagen, 2008)

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column.

Important points before starting

■ The yellow color of Buffer QG indicates a pH ≤7.5.

■ Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to

the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix.

For example, if the agarose gel slice is 100 mg, add 100 μl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube.

Collection tubes are reused to reduce plastic waste.

9. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the centre of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Appendix vi - Polymerase Chain Reaction parameters for amplifying short-tandem repeats and Major Histocompatibility Complex Class II B exon 2 - intron 2 - exon 3

Short-tandem repeats:

1st cycle:	**95ºC**	**5'**	
	95ºC	**1'**	
	55ºC	**10"**	
	72ºC	**2'**	
2nd cycle:	**94ºC**	**20"**	
	55ºC	**10"**	**x15**
	72ºC	**1'**	
3rd cycle:	**92ºC**	**20"**	
	56ºC	**10"**	**x25**
	72ºC	**1'**	
4th cycle	**92ºC**	**20"**	
	56ºC	**10"**	
	72ºC	**20'**	
	4ºC	**HOLD**	

Major Histocompatibility Complex:

Cycle	Temperature	Time	
1st cycle:	**95°C**	**5’**	
	95°C	**1’**	
	59°C	**20”**	
	72°C	**2’**	
2nd cycle:	**94°C**	**20”**	
	59°C	**20”**	**x15**
	72°C	**2’**	
3rd cycle:	**94°C**	**30”**	
	57°C	**20”**	**x40**
	72°C	**2’**	
4th cycle	**94°C**	**30”**	
	57°C	**20”**	
	72°C	**20’**	
	4°C	**HOLD**	

Appendix vii - Protocol for Ligations Using pGEM-T Easy Vector and the 2X Rapid Ligation Buffer (Promega, 2010)

1. Briefly centrifuge the pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.

2. Set up ligation reactions as described below.
Note: Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat.# 20170-310). Vortex the 2X Rapid Ligation Buffer vigorously before each use.

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Reaction Component	**Standard Reaction (µl)**	**Positive Control (µl)**	**Background Control (µl)**
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEM®-T Easy Vector (50ng)	1	1	1
PCR Product	X	-	-
Control Insert DNA	-	2	-
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
Nuclease-free water to a final volume of	**10**	**10**	**10**

*Molar ratio of PCR product:vector may require optimization.

Notes:
1. Use only the T4 DNA Ligase supplied with this system to perform pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
2. 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
3. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.

4. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard®SV Gel and PCR Clean-Up System (Cat.# A9281). Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimer.

Appendix viii - Bioline ElectroSHOX Competent Cells - Suggested Transformation Procedure for Optimal Results - (Bioline, 2013)

1. Pre-chill electroporation cuvettes, electroporation chamber (if applicable), and microcentrifuge tubes on ice.

2. Remove cells from –80°C and thaw on ice.

3. Place 40-50µl of the competent cells into a chilled microcentrifuge tube. Add 1-5µl of sample DNA to cells. Thoroughly mix by gently pipetting and incubate on ice for approximately 1 minute. Note: For optimal results, sample DNA should be in sterile H2O or low ionic strength buffer such as TE. If a control is desired, repeat this step with 2µl of the provided pUC19 in a separate tube. Refreeze any unused cells and store at –80° C.

4. Transfer cell mixture into a pre-chilled cuvette and pulse using settings recommended by manufacturer of electroporator. As a general guideline, maximum transformation efficiency is normally attained using cuvettes with a 0.1 cm gap with an applied voltage of ~1800 (field strength of ~18 kV/cm).

5. Immediately dilute pulsed cells to 1ml with SOC medium and transfer to a sterile culture tube.

6. Gently shake culture tube ~200rpm for 60 minutes at 37°C.

7. Plate by spreading 5-200µl of cell transformation mixture on LB agar plates containing appropriate antibiotic and incubate overnight at 37°C.

Appendix ix - Favorgen FavorPrepPlasmid DNA Extraction Mini Kit (Favorgen, 2011)

Important Notes:

1. Buffer provided in this kit contain irritants. Wear gloves and lab coat when handling these buffer.
2. Brief spin RNase A tube to remove drops from the inside of the lid. Add 1 ml of FAPD1 Buffer into RNase A tube and mix well. Transfer the mixture into FAPD1 Buffer bottle and store at 4 º C.
3. Check FAPD2 Buffer before use. Warm FAPD2 Buffer at 55 º C for 10 minutes if any precipitation formed. Don't shake FAPD2 Buffer vigorously.
4. To avoid acidification of FAPD2 Buffer from CO_2 in the air, close the bottle immediately after use.
5. For FAPDE 001, add 13 ml ethanol (96~100%) to W1 Buffer when first open. For FAPDE 001-1, add 36 ml ethanol (96~100%) to W1 Buffer when first open.
6. For FAPDE 001, add 80 ml ethanol (96~100%) to Wash Buffer when first open. For FAPDE 001-1, add 200 ml ethanol (96~100%) to Wash Buffer when first open.
7. All centrifuge steps are done at full speed (14,000 rpm or 10,000 x g) in a microcentrifuge.

Protocol:

1. Transfer 1-5 ml of well-grown bacteria culture to a microcentrifuge tube (not provided).

2. Descend the bacteria by centrifuging for 1-2 min and discard the supernatant completely.

3. Add 250 µl of FAPD1 Buffer to the pellet and resuspend the cells completely by pipetting.

• Make sure that RNase A has been added into FAPD1 Buffer when first open.

• No cell pellet should be visible after resuspension of the cells.

4. Add 250 µl of FAPD2 Buffer and gently invert the tube 5 times to lyse the cells
and incubate at room temperature for 2 min.

• Do not vortex, vortex may shear genomic DNA. If necessary, continue inverting the tube until the lysate beccome clear.

• Do not proceed this step over 5 min.

5. Add 350 µl of FAPD3 Buffer and invert the tube 5 times immediately but gently.

• Invert immediately after addind FAPD3 Buffer will avoid asymmetric precipitation.

6. Centrifuge for 10 min. During centrifuging, place a FAPD Column in a Collection Tube.

7. Transfer the suspernatant carefully to FAPD Column. Centrifuge for 1 min then discard the flow-through.

• Do not transfer any white pellet into the column.

8. Add 400 µl of W1 Buffer to FAPD Column. Centrifuge for 1 min then discard the flow-through.

• Make sure that ethanol (96-100 %) has been added into W1 Buffer when first open.

9. Add 750 µl of Wash Buffer to FAPD Column. Centrifuge for 1 min then discard the flow-through.

• Make sure that ethanol (96-100 %) has been added into Wash Buffer when first open.

10. Centrifuge for an additional 5 min to dry the column.

• Important step ! This step will remove the residual liquid completely that will inhibit subsequent enzymatic reaction.

11. Place FAPD Column to a new 1.5 ml microcentrifuge tube (not provided).

12. Add 50 µl ~ 100 µl of Elution Buffer or ddH2O to the membrane center of FAPD Column. Stand the column for 1 min.

• Important step ! For effective elution, make sure that the elution solution is dispensed on the membrane center and is absorbed completely.

13. Centrifuge for 1 min to elute plasmid DNA.

14. Store plasmid DNA at 4 °C or -20 °C.

References

Adams MS & Villablanca FX (2007) *Consequences of a genetic bottleneck in California condors: a mitochondrial DNA perspective*, Nuttall Ornithological Club, Cambridge MA & American Ornithologists Union, Washington D.C.

Addison JA & Diamond AW (2011) Population genetics and effective size of the critically endangered Nihoa millerbird (*Acrocephalus familiaris kingi*). *The Auk* **128**, 265-72.

Adlard RD, Peirce MA & Lederer R (2004) Blood parasites of birds from south-east Queensland. *Emu* **104**, 191-6.

Aguilar A, Edwards SV, Smith TB & Wayne RK (2006) Patterns of variation in MHC Class II B Loci of the little greenbul (*Andropadus virens*) with comments on MHC evolution in birds. *Journal of Heredity* **97**, 133-42.

Aguilar A, Roemer G, Debenham S, Binns M, Garcelon D, Wayne RK & Schaal BA (2004) High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3490-4.

Albayrak T, Gonzalez J, Drovetski S & Wink M (2012) Phylogeography and population structure of Krüper's nuthatch *Sitta krueperi* from Turkey based on microsatellites and mitochondrial DNA. *Journal of Ornithology* **153**, 405-11.

Alcaide M, Edwards SV & Negro JJ (2007) Characterization, polymorphism, and evolution of MHC Class II B genes in birds of prey. *Journal of Molecular Evolution* **65**, 541-54.

Alda F, Ruiz-López MJ, García FJ, Gompper ME, Eggert LS & García JT (2013) Genetic evidence for multiple introduction events of raccoons (*Procyon lotor*) in Spain. *Biological Invasions* **15**, 687-98.

Algar DA, Burbidge AA & Angus GJ (2002) Cat eradication on Hermite Island, Montebello Islands, Western Australia. In: *Turning the tide: the eradication of invasive species* (ed. by Clout CVM), IUCN SSC Invasive Species Specialist Group, IUCN, Gland, Switzerland and Cambridge, UK.

Allendorf FW & Luikart G (2007) *Conservation and the genetics of populations*. Blackwell, Malden.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (2007) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–402.

Ambrose SJ, Bradshaw SD, Withers PC & Murphy DP (1996) Water and energy balance of captive and free-ranging spinifexbirds (*Eremiornis carteri*) North (Aves : Sylviidae) on Barrow Island, Western Australia. *Australian Journal of Zoology* **44**, 107-17.

Ambrose SJ & Murphy DP (1994) Synchronous breeding of land birds on Barrow Island, Western Australia, after cyclonic summer rains. *Emu* **94**, 55-8.

Aparicio JM, Ortego J & Cordero PJ (2007) Can a simple algebraic analysis predict markers–genome heterozygosity correlations? *Journal of Heredity* **98**, 93-6.

Arden SL, Ma W, Ewen JG, Armstrong DP & Lambert DM (1997) Social and sexual monogamy in translocated New Zealand robin populations detected using minisatellite DNA. *The Auk* **114**, 120-6.

Ardern SL & Lambert DM (1997) Is the black robin in genetic peril? . *Molecular Ecology* **6**, 21-8.

Armstrong DP & Seddon PJ (2008) Directions in reintroduction biology. *Trends in Ecology and Evolution* **23**, 20-5.

Bain D & French K (2009) Impacts on a threatened bird population of removals for translocation. *Wildlife Research* **36**, 516-21.

Bain MB (2010) Colorado pikeminnow re-introduction and population augmentation in rivers of the Western USA In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 49-52, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Baling M, Wedding C, Barry M, Ji W & Brunton DH (2010) Re-introduction of shore skinks to offshore islands in the Auckland region, New Zealand In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 88-92, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Balloux F (2004) Heterozygote excess in small populations and the heterozygote-excess effective population size. *Evolution* **58**, 1891-900.

Balloux F, Amos W & Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology* **13**, 3021-31.

Balvanera P, Pfisterer AB, Buchmann N, He J-S, Nakashizuka T, Raffaelli D & Schmid B (2006) Quantifying the evidence for biodiversity effects on ecosystem functioning and services. *Ecology Letters* **9**, 1146-56.

Bangs EE & Smith DW (2008) Re-introduction of the gray wolf into Yellowstone National Park and central Idaho, USA In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 167-71, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Barker FK, Cibois A, Schikler P, Feinstein J & Cracraft J (2004) Phylogeny and diversification of the largest avian radiation. *Proceedings of the National Academy of Science of the United States of America* **101**, 11040-5.

Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, Marshall C, McGuire JL, Lindsey EL, Maguire KC, Mersey B & Ferrer EA (2011) Has the Earth's sixth mass extinction already arrived? *Nature* **471**, 51-7.

Barrett S, Dillon R, Cochrane A & Monks L (2011) Conservation introduction of a threatened narrow range endemic species, *Banksia montana*, from the Stirling Range National Park, southern Western Australia In: *Global Re-introduction Perspectives: More case studies from around the globe* (ed. by P Soorae), pp. 220-3, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Beissinger SR, Wunderle Jr. JM, Meyers JM, Sæther BE & Engen S (2008) Anatomy of a bottleneck: diagnosing factors limiting population growth in the Puerto Rican parrot. *Ecological Monographs* **78**, 185-203.

Biedrzycka A, Kloch A, Buczek M & Radwan J (2011) Major histocompatibility complex DRB genes and blood parasite loads in fragmented populations of the spotted suslik *Spermophilus suslicus*. *Mammalian Biology* **76**, 672-7.

Blackburn TM, Cassey P, Duncan RP, Evans KL & Gaston KJ (2004) Avian extinction and mammalian introductions on oceanic islands. *Science* **305**, 1955-8.

Bollmer JL, Dunn PO, Whittingham LA & Wimpee C (2010) Extensive MHC Class II B gene duplication in a passerine, the common yellowthroat (*Geothlypis trichas*). *Journal of Heredity* **101**, 448-60.

Bollmer JL, Hull JM, Ernest HB, Sarasola JH & Parker PG (2011) Reduced MHC and neutral variation in the Galápagos hawk, an island endemic. *BMC Evolutionary Biology* **11**.

Bollmer JL, Whiteman NK, Cannon MD, Bednarz JC, de Vries T & Parker PG (2005) Population genetics of the Galápagos hawk (*Buteo galapagoensis*): genetic monomorphism within isolated populations. *The Auk* **122**, 1210-24.

Bonneaud C, Sorci G, Morin V, Westerdahl H, Zoorob R & Wittzell H (2004) Diversity of MHC Class I and IIB genes in house sparrows (*Passer domesticus*). *Immunogenetics* **55**, 855-65.

Boon WM, Chambers GK & Daugherty CH (1999) Genetic analysis of Forbes' parakeet (*Cyanoramphus forbesi*). *Conservation Advisory Science Notes No.262*, Department of Conservation, Wellington.

Bouzat JL, Johnson JA, Toepfer JE, Simpson SA, Esker TL & Westemeier RL (2009) Beyond the beneficial effects of translocations as an effective tool for the genetic restoration of isolated populations. *Conservation Genetics* **10**, 191-201.

Bowie MH (2010a) Re-introduction of the leaf-vein slug to Quail Island, Banks Peninsula, Canterbury, New Zealand In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 6-8, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi

Bowie MH (2010b) Re-introduction of the Banks Peninsula tree weta to Quail Island, Banks Peninsula, Canterbury, New Zealand In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 9-11, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Brekke P, Bennett PM, Santure AW & Ewen JG (2011) High genetic diversity in the remnant island population of hihi and the genetic consequences of re-introduction. *Molecular Ecology* **20**, 29-45.

Briskie JV & Mackintosh M (2004) Hatching failure increases with severity of population bottlenecks in birds. *Proceedings of the National Academy of Science of the United States of America* **101**, 558-61.

Brook BW, Tonkyn DW, O'Grady JJ & Frankham R (2002) Contribution of inbreeding to extinction risk in threatened species. *Conservation Ecology* **6**, 16.

Brouwer L, Komdeur J & Richardson DS (2007) Heterozygosity–fitness correlations in a bottlenecked island species: a case study on the Seychelles warbler. *Molecular Ecology* **16**, 3134-44.

Bruford MW & Wayne RK (1993) Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development* **3**, 939-43.

Burbidge A, Comer S & Danks A (2005) Threatened birds and wildfire in south-west Western Australia. *Wingspan* **Supplement 15**, 18-20.

Burbidge AA (2004) Montebello Renewal: Western Shield review—February 2003. *Conservation Science Western Australia* **5**, 194-201.

Burbidge AA, Blyth JD, Fuller PJ, Kendrick PG, Stanley FJ & Smith LA (2000) The terrestrial vertebrate fauna of the Montebello Islands, Western Australia. *CALMScience* **3**, 95-107.

Burbidge AA, Folley GL & Smith GT (1986) The Noisy Scrub-bird. Western Australian Wildlife Management Program No. 2, Department of Conservation & Land Management, Como.

Burri R, Niculita-Hirzel H, Roulin A & Fumagalli L (2008) Isolation and characterization of major histocompatibility complex (MHC) class II B genes in the Barn owl (Aves: *Tyto alba*). *Immunogenetics* **60**, 543-50.

Burri R, Promerová M, Goebel J & Fumagalli L (2014) PCR-based isolation of multigene families: lessons from the avian MHC class IIB. *Molecular Ecology Resources* **14**, 778-88.

Canal D, Alcaide M, Anmarkrud JA & Potti J (2010) Towards the simplification of MHC typing protocols: targeting classical MHC class II genes in a passerine, the pied flycatcher *Ficedula hypoleuca*. *BMC Research Notes* **3**, 236.

Cardinale BJ, Duffy JE, Gonzalez A, Hooper DU, Perrings C, Venail P, Narwani A, Mace GM, Tilman D, Wardle DA, Kinzig AP, Daily GC, Loreau M, Grace JB, Larigauderie A, Srivastava DS & Naeem S (2012) Biodiversity loss and its impact on humanity. *Nature* **486**, 59-67.

Cardoso MJ, Eldridge MDB, Oakwood M, Rankmore B, Sherwin WB & Firestone KB (2009) Effects of founder events on the genetic variation of translocated island populations: implications for conservation management of the northern quoll. *Conservation Genetics* **10**, 1719-33.

Case TJ, Bolger DT & Richman AD (1992) Reptilian extinctions: the last ten thousand years. In: *Conservation biology: the theory and practice of nature conservation, preservation and management* (ed. by Jain PFS), pp. 90-125, Chapman and Hall, New York.

Castalanelli MA, Severtson DL, Brumley CJ, Szito A, Foottit RG, Grimm M, Munyard K & Groth DM (2010) A rapid non-destructive DNA extraction method for insects and other arthropods. *Journal of Asia Pacific Entomology* **13**, 243-8.

Castoe TA, Poole AW, Gu W, de Koning APJ, Daza JM, Smith EN & Pollock DD (2010) Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence. *Molecular Ecology Resources* **10**, 341-7.

Castro-Prieto A, Wachter B & Sommer S (2011) Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. *Molecular Biology and Evolution* **28**, 1455-68.

Chan C-H, Ballantyne KN, Aikman H, Fastier D, Daugherty CH & Chambers GK (2006) Genetic analysis of interspecific hybridisation in the world's only Forbes' parakeet (*Cyanoramphus forbesi*) natural population. *Conservation Genetics* **7**, 493-506.

Chan C-H, Zhao Y & Chambers GK (2009) Microsatellite DNA markers provide informative genetic data for studies on New Zealand *Cyanoramphus* parakeets. *New Zealand Natural Sciences* **34**, 69-76.

Chan C-H, Robertson HA, Saul EK, Nia LV, Phuong Luong V, Kong X, Zhao Y & Chambers GK (2011) Genetic variation in the kakerori (*Pomarea dimidiata*), an endangered endemic bird successfully recovering in the Cook Islands. *Conservation Genetics* **12**, 441-7.

Chesser RT & ten Have J (2007) On the phylogenetic position of the scrub-birds (Passeriformes:Menurae: Atrichornithidae) of Australia. *Journal of Ornithology* **148**, 471-6.

Christidis L & Norman JA (2010) Evolution of the Australasian songbird fauna. *Emu*, **110**, 21-31.

Coico R, Sunshine G & Benjamini E (2003) *Immunology: a short course*. John Wiley & Sons, Inc., Hoboken NJ.

Comer S & Danks A (2000) Noisy scrub-bird recovery plan annual report 1999. South Coast Threatened Birds Recovery Team, Albany.

Comer S, Danks A, Burbidge AH & Tiller C (2010) The history and success of noisy scrub-bird re-introductions in Western Australia: 1983-2005 In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 187-92, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Conant S & Morin M (2001) Why isn't the Nihoa millerbird extinct? *Studies in Avian Biology* **22**, 338-46.

Cornuet JM & Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-14.

Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neill RV, Paruelo J, Raskin RG, Sutton P & van den Belt M (1997) The value of the world's ecosystem services and natural capital. *Nature* **387**, 253-60.

Cramer ERA, Stenzler L, Talaba AL, Makarewich CA, Vehrencamp SL & Lovette IJ (2008) Isolation and characterization of SNP variation at 90 anonymous loci in the banded wren (*Thryothorus pleurostictus*). *Conservation Genetics* **9**, 1657-60.

Crow JF & Kimura M (1970) *An introduction to population genetics theory*, Burgess Publishing Co.

Csencsics D, Brodbeck S & Holderegger R (2010) Cost-effective, species-specific microsatellite development for the endangered dwarf bulrush (*Typha minima*) using next-generation sequencing technology. *Journal of Heredity* **101**, 789-93.

Culver M, Hedrick PW, Murphy K, O'Brien S & Hornocker MG (2008) Estimation of the bottleneck size in Florida panthers. *Animal Conservation* **11**, 104-10.

Daltry JC, Anthonyson D & Morton MN (2010) Re-introduction of the Antiguan racer to offshore islands of Antigua, West Indies In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 98-103, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Danks A (1994) Noisy scrub-bird translocations: 1983-1992. In: *Reintroduction Biology of Australian and New Zealand Fauna* (ed. by Serena M), pp. 129-34, Surrey Beatty & Sons., Sydney.

Danks A (1997) Conservation of the Noisy scrub-bird: a review of 35 years of research and management. In: *Pacific Conservation Biology* (pp. 341-9, Surrey Beatty & Sons., Sydney.

Danks A, Burbidge AA, Burbidge AH & Smith GT (1996) Noisy scrub-bird recovery plan. WA Department of Conservation and Land Management, Perth.

Darwin CR (1872) *On the origin of species by means of natural selection* or *the preservation of favoured races in the struggle for life*, Arcturus Publishing Limited, London.

De Barba M, Waits LP, Garton EO, Genovesi P, Randi E, Mustoni A & Groffs C (2010) The power of genetic monitoring for studying demography, ecology and genetics of a reintroduced brown bear population. *Molecular Ecology* **19**, 3938-51.

DeWoody YD & DeWoody JA (2005) On the Estimation of Genome-wide Heterozygosity Using Molecular Markers. *Journal of Heredity* **96**, 85-8.

DPaW (2014) South Coast threatened birds recovery plan. Department of Parks and Wildlife, Perth, Western Australia.

Diamond JM (1984) "Normal" extinctions of isolated populations. In: *Extinctions* (ed. by Nitecki M), University of Chicago Press, Chicago.

Diamond JM, Ashmole NP & Purves PE (1989) The present, past and future of human-caused extinctions [and discussion]. *Philosophical Transactions of the Royal Society London B* **325**, 469-77.

Dirzo R & Raven PH (2003) Global state of biodiversity and loss. *Annual Review of Environment and Resources* **28**, 137-67.

Dixon B & Krauss S (2008) Translocation of the Corrigin grevillea in south Western Australia In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 229-34, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ & Ovenden JR (2014) NeEstimator V2: re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Molecular Ecology Resources* **14**, 209-14.

Donald PF, Collar NJ, Marsden SJ & Pain DJ (2010) *Facing Extinction: the world's rarest birds and the race to save them*, T&AD Poyser, London.

Dovc A, Dovc P, Kese D, Vlahović K, Pavlak M & Zorman-Rojs O (2005) Long-term study of Chlamydophilosis in Slovenia. *Veterinary Research Communication* **29**, 23-6.

Driskell AC, Pruett-Jones S, Tarvin KA & Hagevik S (2002) Evolutionary relationships among blue- and black-plumaged populations of the white-winged fairy-wren (*Malurus leucopterus*). *Australian Journal of Zoology* **50**, 581-95.

Driskell AC, Prum RO & Pruett-Jones S (2010) The evolution of black plumage from blue in Australian fairy-wrens (Maluridae): genetic and structural evidence. *Journal of Avian Biology* **41**, 505-14.

Drouin G, Prat F, Ell M & Paul Clarke GD (1999) Detecting and Characterizing Gene Conversions Between Multigene Family Members. *Molecular Biology and Evolution* **16**, 1369-90.

Du H, Zheng J, Wu M, Zhao Q & Wang D (2010) High MHC DQB variation and asymmetric allelic distribution in the endangered Yangtze finless porpoise, *Neophocaena phocaenoides asiaeorientalis*. *Biochemical Genetics* **48**, 433-49.

Duffy JE (2009) Why biodiversity is important to the functioning of real-world ecosystems. *Frontiers in Ecology and the Environment* **7**, 437-44.

Duncan RP & Blackburn TM (2004) Extinction and endemism in the New Zealand avifauna. *Global Ecology and Biogeography* **13**, 509-17.

Duncan RP, Blackburn TM & Worthy TH (2002) Prehistoric bird extinctions and human hunting. *Proceedings of the Royal Society B: Biological Sciences* **269**, 517-21.

Dunn PO, Bollmer JL, Freeman-Gallant CR & Whittingham LA (2001) MHC variation is related to a sexually selected ornament, survival, and parasite resistance in common yellowthroats. *Evolution* **67**, 679-87.

Eckert KA, Mowery A & Hile SE (2002) Misalignment-mediated DNA polymerase beta mutations: comparison of microsatellite and frame-shift error rates using a forward mutation assay. *Biochemistry (Mosc.)* **41**, 10490-8.

Edmonds D (2007) Health screening of the endangered Noisy Scrub-bird (*Atrichornis clamosus*). In: *Vet 639 Field Project Final Report*, Unpublished.

Eimes JA, Bollmer JL, Dunn PO, Whittingham LA & Wimpee C (2010) MHC Class II diversity and balancing selection in greater prairie-chickens. *Genetica* **138**, 265-71.

Eizaguirre C, Yeates SE, Lenz TL, Kalbe M & Milinski M (2009) MHC-based mate choice combines good genes and maintenance of MHC polymorphism. *Molecular Ecology* **18**, 3316-29.

Ejsmond MJ & Radwan J (2011) MHC diversity in bottlenecked populations: a simulation model. *Conservation Genetics* **12**, 129-37.

Ellegren H (2004) Microsatellites: simple sequences with complex evolution. *Nature Reviews* **5**, 435-45.

Emslie RH, Amin R & Kock R (2009) *Guidelines for the in situ re-introduction and translocation of African and Asian rhinoceros*. IUCN, Gland, Switzerland.

En LI, Wu XB & Yan P (2006) Genetic variation and trans-species polymorphism of MHC Class II B genes in reptiles. *Asiatic Herpetologial Research* **11**, 50-6.

Evans SR & Sheldon BC (2008) Interspecific patterns of genetic diversity in birds: correlations with extinction risk. *Conservation Biology* **22**, 1016-25.

Fahlén J (2014) SNP-based conservation genetics of the southern Swedish brown bear (*Ursus arctos*). In: *Department of Wildlife, Fish and Environmental Studies, Umeå*, Masters Thesis.

Faircloth BC (2008) MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* **8**, 92-4.

Farell EM & Alexandre G (2012) Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. *BMC Research Notes* **5**, 257.

Farmer C, Kohley R, Freifeld H & Plentovich S (2011) Nihoa millerbird (*Acrocephalus familaris kingi*) translocation protocols (final). (ed. by U.S. Fish and Wildlife Service H, Hawai'i).

Fleischer RC, Slikas B, Beadell J, Atkins C, McIntosh CE & Conant S (2007) Genetic variability and taxonomic status of the Nihoa and Laysan millerbirds. *The Condor* **109**, 954-62.

Fleischer RC, Tarr CL & Pratt TK (1994) Genetic structure and mating system in the palila, an endangered Hawaiian honeycreeper, as assessed by DNA fingerprinting. *Molecular Ecology* **3**, 383-92.

Flesness NR (1989) Mammalian extinction rates: background to the black-footed ferret drama. In: *Conservation biology of the black-footed ferret* (ed. by US Seal ET, MA Bogan & SH Anderson), Yale University Press, New Haven, CT.

Foerster K, Delhey K, Johnsen A, Lifjeld JT & Kempenaers B (2003) Females increase offspring heterozygosity and fitness through extra-pair matings. *Nature* **425**, 714-7.

Frankel OH (1974) Genetic Conservation: our evolutionary responsibility. *Genetics* **78**, 53-65.

Frankham R (1997) Do island populations have less genetic variation than mainland populations? *Heredity* **78**, 311-27.

Frankham R (1998) Inbreeding and extinction: island populations. *Conservation Biology* **12**, 665-75.

Frankham R (2005) Genetics and extinction. *Biological Conservation* **126**, 131-40.

Franklin IR (1980) Evolutionary change in small populations. In: *Conservation biology: an evolutionary-ecological perspective* (ed. by Wilcox MSB), Sinauer Associates, Sunderland, MA.

Fuerst PA & Maruyama T (1986) Considerations on the conservation of alleles and of genic heterozygosity in small managed populations *Zoo Biology* **5**, 171-9.

Galtier N, Nabholz B, Glémin S & Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal *Molecular Ecology* **18**, 4541-50.

Garnett ST, Szabo JK & Dutson G (2011) *The action plan for Australian birds 2010*, Birds Australia. CSIRO Publishing, Collingwood.

Garza JC & Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* **10**, 305-18.

Gautschi B, Widmer A, Joshi J & Koella JC (2002) Increased frequency of scale anomalies and loss of genetic variation in serially bottlenecked populations of the dice snake, *Natrix tessellata*. *Conservation Genetics* **3**, 235-45.

Genovart M, Juste J, Contreras-Díaz & Oro D (2012) Genetic and phenotypic differentiation between the critically endangered Balearic shearwater and neighboring colonies of its sibling species. *Journal of Heredity* **103**, 330-41.

Geyer CJ, Ryder OA, Chemnick LG & Thompson EA (1993) Analysis of relatedness in the California condors, from DNA fingerprints. *Molecular Biology and Evolution* **10**, 571-89.

Gilles, A, Meglécz E, Pech N, Ferreira S, Malausa T & Martin JF (2011) Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing. *BMC Genomics* **12**, 245.

Glenn TC (2011) Field guide to next-generation DNA sequencers. *Molecular Ecology Resources* **11**, 759-69.

Glenn TC, Ojerio RS, Stephan W & Braun MJ (1997) Microsatellite DNA loci for genetic studies of cranes. In: *Proceedings of the 7th North American crane workshop* (ed. by RP Urbanek & DW Stahlecker), pp 36-45, North American Crane Working Group, Grand Island, Nebraska.

Glenn TC & Schable NA (2005) *Isolating Microsatellite DNA Loci*, Elsevier Inc.

Glenn TC, Stephan W & Braun MJ (1999) Effects of a population bottleneck on whooping crane mitochondrial DNA variation. *Conservation Biology* **13**, 1097-107.

Goldstein DB & Schlötterer C (1999) *Microsatellites: evolution and applications*. Oxford University Press, Oxford.

Goren M (2010) Re-introduction of the "extinct in the wild" Yarqon bleak, Israel. In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from*

around the globe (ed. by P Soorae), pp. 45-8, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Griffith B, Scott JM, Carpenter JW & Reed C (1989) Translocation as a Species Conservation Tool: Status and Strategy. *Science* **245**, 477-80.

Griffith SC (2000) High fidelity on islands: a comparative study of extrapair paternity in passerine birds. *Behavioral Ecology* **11**, 265-73.

Griffiths RA, McGrath A & Buckley J (2010) Re-introduction of the natterjack toad in the UK. In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 62-5, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Groombridge JJ, Jones CG, Bruford MW & Nichols RA (2000) 'Ghost' alleles of the Mauritius kestrel. *Nature* **403**, 615.

Grueber CE & Jamieson IG (2011) Low genetic diversity and small population size of takahe *Porphyrio hochstetteri* on European arrival in New Zealand. *Ibis* **153**, 384-94.

Grueber CE, Wallis GP, King T, Jamieson IG (2012) Variation at innative immunity Toll-like receptor genes in a bottlenecked population of a New Zealand robin. *PLoS One* **7**, e45011.

Grueber CE, Knafler GJ, King TM, Senior AM, Grosser S, Robertson B, Weston KA, Brekke P, Harris CLW & Jamieson IG (2015) Toll-like receptor in 10 threatened bird species: relationship with microsatellite heterozygosity. *Conservation Genetics* **16**, 595-611.

Gutierrez-Espeleta GA, Hedrick PW, Kalinowski ST, Garrigan D & Boyce WM (2001) Is the decline of desert bighorn sheep from infectious disease the result of low MHC variation? *Heredity* **86**, 439-50.

Haig SM & Ballou JD (1995) Genetic Diversity in Two Avian Species Formerly Endemic to Guam. *The Auk* **112**, 445-55.

Hair JD & Forrester DJ (1970) The helminth parasites of the starling (*Sturnus vulgaris* L.): a checklist and analysis. *American Midland Naturalist* **83**, 555-64.

Hale KA & Briskie JV (2007) Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation* **10**, 2-10.

Hale ML, Burg TM & Steeves TE (2012) Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies *PLOS One* **7**, e45170.

Hancock JM (1995) The contribution of slippage-like processes to genome evolution. *Journal of Molecular Evolution* **41**, 1038-47.

Hansson B & Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology* **11**, 2467-74.

Hawley DM & Fleischer RC (2012) Contrasting epidemic histories reveal pathogen-mediated balancing selection on Class II MHC diversity in a wild songbird. *PLOS One* **7**, e30222.

He L-P, Wan Q-H, Fang S-G & Xi Y-M (2006) Development of novel microsatellite loci and assessment of genetic diversity in the endangered crested ibis, *Nipponia Nippon*. *Conservation Genetics* **7**, 157-60.

Heber S & Briskie JV (2013) Survival of South Island robins (*Petroica australis*) after a reciprocal translocation to reduce inbreeding depression. *Notornis* **60**, 117-9.

Hedrick P (2005) ‘Genetic restoration:’ a more comprehensive perspective than ‘genetic rescue’. *Trends in Ecology and Evolution* **20**, 109.

Hedrick PW (1995) Gene flow and genetic restoration: the Florida panther as a case study. *Conservation Biology* **9**, 996-1007.

Hedrick PW (2001) Conservation genetics: where are we now? *Trends in Ecology and Evolution* **16**, 629-36.

Hedrick PW, Parker KM, Gutiérrez-Espeleta GA, Rattink A & Lievers K (2000) major histocompatibility complex variation in the Arabian oryx. *Evolution* **54**, 2145-51.

Hedrick PW, Parker KM, Miller EL & Miller PS (1999) Major Histocompatibility Complex variation in the endangered Przewalski's horse. *Genetics* **152**, 1701-10.

Helbig M, Theus M & Urbanek S (2005) JGR: Java GUI for R. *Statistical Computing & Graphics* **16**, 9-12.

Hess CM & Edwards SV (2002) The evolution of the major histocompatibility complex in birds. *BioScience* **52**, 423-31.

Hicks JF, Rachlow JL, Rhodes Jr. OE, Williams CL & Waits LP (2007) Reintroduction and genetic structure: Rocky Mountain elk in Yellowstone and the western states. *Journal of Mammalogy* **88**, 129-138.

Hoelzel AR, Stephens JC & O'Brien SJ (1999) Molecular genetic diversity and evolution at the MHC DQB locus in four species of pinnipeds. *Molecular Biology and Evolution* **16**, 611-8.

Holderegger R, Kamm U & Gugerli F (2006) Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology* **21**, 797-807.

Holzinger-Umlauf HA-M, Marschang RE, Gravendyck M & Kaleta EF (1997) Investigation on the frequency of *Chlamydia* sp. infections in tits (Paridae). *Avian Pathology* **26**, 779-89.

Holzman DC (2012) Accounting for nature's benefits: the dollar value of ecosystem services. *Environmental Health Perspectives* **120**, 152-7.

Hooper DU, Adair EC, Cardinale BJ, Byrnes JEK, Hungate BA, Matulich KL, Gonzalez A, Duffy JE, Gamfeldt L & O'Connor MI (2012) A global synthesis reveals biodiversity loss as a major driver of ecosystem change. *Nature* **486**, 105-9.

Hughes AL & Nei Ml (1989) Nucleotide substitution at major histocompatibility complex class II loci: Evidence for overdominant selection. *Proceedings of the National Academy of Science of the United States of America* **86**, 958-62.

Hume JP & Walters M (2012) *Extinct Birds*, T & AD Poyser, Bloomsbury, London.

Ingvarsson PK (2001) Restoration of genetic variation lost – the genetic rescue hypothesis. *Trends in Ecology and Evolution* **16**, 62-3.

IUCN (2012) IUCN Red list of threatened species. Version 2012.1. URL http://www.iucnredlist.org.

Ishtiaq F, Dutta S, Yumnam B & Jhala YV (2011) Low genetic diversity in the endangered great Indian bustard (*Ardeotis nigriceps*) across India and implications for conservation. *Conservation Genetics* **12**, 857-63.

Jamieson IG (2007) Has the debate over genetics and extinction of island endemics truly been resolved? *Animal Conservation* **10**, 139-44.

Jamieson IG (2009) Loss of genetic diversity and inbreeding in New Zealand's threatened bird species In: *Science for Conservation 293* p. 59. Department of Conservation, Wellington.

Janeway CA, Travers Jr P, Walport M & Shlomchik MJ (2001) *Immunobiology: the immune system in health and disease 5th edition*, Garland Science, New York.

Jansen van Rensburg A, Bloomer P, Ryan PG & Hansson B (2012) Ancestral polymorphism at the major histocompatibility complex (MHCIIß) in the *Nesospiza* bunting species complex and its sister species (*Rowettia goughensis*). *BMC Evolutionary Biology* **12**, 143.

Jarvi SI, Tarr CL, Mcintosh CE, Atkinson CT & Fleischer RC (2001) Natural selection of the major histocompatibility complex (MHC) in Hawaiian honeycreepers (Drepanidinae). *Molecular Ecology* **13**, 2157-68.

Jeffrey KJM & Bangham CRM (2000) Do infectious diseases drive MHC diversity? *Microbes and Infection* **2**, 1335-41.

Jepson A, Banya W, Sisay-Joof F, Hassan-King M, Nunes C, Bennett S & Whittle H (1997) Quantification of the relative contribution of major histocompatibility complex (MHC) and non-MHC genes to human immune responses to foreign antigens. *Infection and Immunity* **65**, 872-6.

Jin L, Zhong Y & Chakraborty R (1994) The exact numbers of possible microsatellite motifs. *American Journal of Human Genetics* **55**, 582-3.

Johnsen A, Andersen V, Sunding C & Lifjeld JT (2000) Female bluethroats enhance offspring immunocompetence through extra-pair copulations. *Nature* **406**, 296-9.

Johnson JA, Tingay RE, Culver M, Hailer F, Clarke ML & Mindell DP (2009) Long-term survival despite low genetic diversity in the critically endangered Madagascar fish-eagle. *Molecular Ecology* **18**, 54-63.

Johnson JA, Toepfer JE & Dunn PO (2003) Contrasting patterns of mitochondrial and microsatellite population structure in fragmented populations of greater prairie-chickens. *Molecular Ecology* **12**, 3335-47.

Johnson TH & Stattersfield AJ (1990) A global review of island endemic birds. *Ibis* **132**, 167-80.

Johnson WE, Onorato DP, Roelke ME, Land ED, Cunningham M, Belden RC, McBride R, Jansen D, Lotz M, Shindle D, Howard J, Wildt DE, Penfold LM, Hostetler JA, Oli MK & O'Brien SJ (2010) Genetic restoration of the Florida panther. *Science* **329**, 1641-5.

Jones CG & Merton DV (2012) A tale of two islands: the rescue and recovery of endemic birds in New Zealand and Mauritius. In: *Reintroduction Biology: Integrating Science and Management* (ed. by JG Ewen DP Armstrong, KA Parker & PJ Seddon), Blackwell Publishing Ltd.

Kaeuffer R, Réale D, Coltman DW & Pontier D (2007) Detecting population structure using STRUCTURE software: effect of background linkage disequilibrium. *Heredity* **99**, 374-80.

Kalbe M, Eizaguirre C, Dankert I, Reusch TBH, Sommerfeld RD, Wegner KM & Milinski M (2009) Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. *Proceedings of the Royal Society B: Biological Sciences* **276**, 925-34.

Kalinowski ST (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes* **5**, 187-9.

Kalinowski ST (2011) The computer program STRUCTURE does not reliably identify the main genetic clusters within species: simulations and implications for human population structure. *Heredity* **106**, 625-32.

Kalinowski ST, Muhlfeld CC, Guy CS & Cox B (2010) Founding population size of an aquatic invasive species. *Conservation Genetics* **11**, 2049-53.

Kamiya T, O'Dwyer K, Westerdahl H, Senior A & Nakagawa S (2014) A quantitative review of MHC-based mating preference: the role of diversity and dissimiliarity. *Molecular Ecology* **23**, 5151-63.

Karunakaran K, Yu H, Jiang X, Moon K-M, Shen C, Foster L & Brunham R (2012) MHC Class II immunoproteomic analysis of *Chlamydia trachomatis* and *C. muridarum* infected murine dendritic cells reveals overlapping immunodominant epitopes. *Journal of Immunology* **188** (Meeting Abstract Supplement) 106.7.

Keller LF & Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecology and Evolution* **17**, 230-41.

Kibbe WA (2007) OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research* **35**, W43-W6.

King AG, Cooke GM, Johnson RN & Major RE (2012) Development and multiplex genotyping of eighteen novel microsatellite markers for a threatened saltmarsh bird *Epthianura albifrons* (Meliphagidae). *Conservation Genetics Resources* **4**, 893-6.

Kiwan K, El AlKamy H & Al Daheri A (2008) Re-introduction of Arabian oryx into Um Al Zomoul, Abu Dhabi Emirate, United Arab Emirates In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 177-80, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Kryazhimskiy S & Plotkin, JB (2008) The population genetics of dN/dS. *PLoS Genetics* **4**, e1000304.

Lambert DM, King T, Shepherd LD, Livingston A, Anderson S & Craig JL (2005) Serial population bottlenecks and genetic variation: Translocated populations of the New Zealand saddleback (*Philesturnus carunculatus rufusater*). *Conservation Genetics* **6**, 1-14.

Latta, RG (1998) Differentiation of allelic frequencies at quantative trait loci affecting locally adaptive traits. *The American Naturalist* **151**, 283-92.

Lau MWN & Banks C (2008) Translocation of Romer's tree frog in Hong Kong SAR, China In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 47-9, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Lawrence HA, Taylor GA, Millar CD & Lambert DM (2008) High mitochondrial and nuclear genetic diversity in one of the world's most endangered seabirds, the Chatham Island taiko (*Pterodroma magentae*). *Conservation Genetics* **9**, 1293-301.

Leberg PL & Firmin BD (2008) Role of inbreeding depression and purging in captive breeding and restoration programmes. *Molecular Ecology* **17**, 334-43.

Lindsay DL, Barr KR, Lance RF, Tweddale SA, Hayden TJ & Leberg PL (2008) Habitat fragmentation and genetic diversity of an endangered, migratory songbird, the golden-cheeked warbler (*Dendroica chrysoparia*). *Molecular Ecology* **17**, 2122-33.

Liu K & Muse SV (2005) PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* **21**, 2128-9.

Longmire JL, Hahn DC & Roach JL (1999) Low abundance of microsatellite repeats in the genome of the brown-headed cowbird (*Molothrus ater*). **90**, 574-8.

Lowe A, Harris S & Ashton P (2004) *Ecological genetics: design analysis and application*, Blackwell Science Ltd, Malden, MA.

Luikart G & Cornuet J-M (1999) Estimating the effective number of breeders from heterozygote excess in progeny. *Genetics* **151**, 1211-6.

MacArthur RH & Wilson EO (1967) *The theory of island biogeography*. Princeton University Press.

Martínez-Cruz B, Godoy JA & Negro JJ (2004) Population genetics after fragmentation: the case of the endangered Spanish imperial eagle (*Aquila adalberti*). *Molecular Ecology* **13**, 2243-55.

Maruyama T & Nei M (1981) Genetic variability maintained by mutation and overdominant selection in finite populations. *Genetics* **98**, 441-59.

Mason RAB, Browning TL & Eldridge MDB (2011) Reduced MHC Class II diversity in island compared to mainland populations of the black-footed rock-wallaby (*Petrogale lateralis lateralis*). *Conservation Genetics* **12**, 91-103.

McCairns RJS, Bourget S & Bernatchez L (2011) Putative causes and consequences of MHC variation within and between locally adapted stickleback demes. *Molecular Ecology* **20**, 486-502.

McClelland EE, Penn DJ & Potts WK (2003) Major histocompatibility complex heterozygote superiority during coinfection. *Infection and Immunity* **71**, 2079-86.

McLoughlin J (2003) Microsatellite diversity of the three sub-populations of the vulnerable noisy scrub-bird (*Atrichornis clamosus*) in Western Australia. In: *School of Biomedical Sciences, Curtin University of Technology*, Unpublished Honours Thesis.

Meglécz E, Neve G, Biffin, E & Gardner MG (2012) Breakdown of phylogenetic signal: a survey of microsatellite densities in 454 shotgun sequences from 154 non model Eukaryote species. *PLoS One* **7**, 1-15.

Menotti-Raymond, MA & O'Brien, SJ (1995) Evolutionary conservation of ten microsatellite loci in four species of Felidae *Journal of Heredity* **86**, 319-22.

Miller HC, Bowker-Wright G, Kharkrang M & Ramstad K (2011) Characterisation of Class II B MHC genes from a ratite bird, the little spotted kiwi (*Apteryx owenii*). *Immunogenetics* **63**, 223-33.

Miller HC & Lambert DM (2004a) Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). *Molecular Ecology* **13**, 3709-21.

Miller HC & Lambert DM (2004b) Gene duplication and gene conversion in class II MHC genes of New Zealand robins (Petroicidae). *Immunogenetics* **56**, 178-91.

Miller HC, Lambert DM, Millar CD, Robertson BC & Minot EO (2003) Minisatellite DNA profiling detects lineages and parentage in the endangered kakapo (*Strigops habroptilus*) despite low microsatellite DNA variation. *Conservation Genetics* **4**, 265-74.

Milot E, Weimerskirch H, Duchesne P & Bernatchez L (2007) Surviving with low genetic diversity: the case of albatrosses. *Proceedings of the Royal Society B: Biological Sciences* **274**, 779-87.

Mindell DP (1997) *Avian molecular evolution and systematics*, Academic Press, San Diego, CA.

Mock KE, Latch EK & Rhodes Jr. OE (2004) Assessing losses of genetic diversity due to translocation: long-term case histories in Merriam's turkey (*Meleagris gallopavo merriami*). *Conservation Genetics* **5**, 631-45.

Morin PA, Luikart G, Wayne RK & the SNP workshop group (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology and Evolution* **19**, 208-16.

Morin PA, Martien KK & Taylor BL (2009) Assessing statistical power of SNPs for population structure and conservation studies. *Molecular Ecology Resources* **9**, 66-73.

Morris-Pocock JA, Hennicke JC & Friesen VL (2012) Effects of long-term isolation on genetic variation and within-island population genetic structure in Christmas Island (Indian Ocean) seabirds. *Conservation Genetics* **13**, 1469-81.

Mulder RA, Dunn PO, Cockburn A, Lazenby-Cohen KA & Howell MJ (1994) Helpers liberate female fairy-wrens from constraints on extra-pair mate choice. *Proceedings: Biological Sciences* **255**, 223-9.

Munyard KA, Ledger JM, Lee CY, Babra C & Groth DM (2009) Characterization and multiplex genotyping of alpaca tetranucleotide microsatellite markers. *Small Ruminant Research* **85**, 153-6.

Murn C, King A, Hunt S & Smith A (2008) Captive breeding and re-introduction of red kites to Hampshire, England In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 159-62, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Myers N (1997) The world's forest and their ecosystem services. In: *Nature's Services: Societal Dependence On Natural Ecosystems* (ed. by Daily G), Island Press, Washington DC.

Nei M, Maruyama T & Chakraborty R (1975) Bottleneck effect and genetic-variability in populations. *Evolution* **29**, 1-10.

Netea MG, Wijmenga C & O'Neill LAJ (2012) Genetic variatin in Toll-like receptors and disease susceptibility. *Nature Immunology* **13**, 535-42.

Nomura T (2008) Estimation of effective number of breeders from molecular coancestry of single cohort sample. *Evolutionary Applications* **1**, 462-74.

Nunney L (1993) The influence of mating system and overlapping generations on effective population size. *Evolution* **47**, 1329-41.

O'Brien SJ, Wildt DE, Bush M, Caro TM, FitzGibbon C, Aggundey I & Leakey RE (1987) East African cheetahs: evidence for two population bottlenecks? *Proceedings of the National Academy of Science of the United States of America* **84**, 508-11.

O'Brien S, Wildt DE, Goldman D, Merril CR & Bush M (1983) The cheetah is depauperate in genetic variation. *Science* **221**, 459-62.

O'Brien SJ & Everman JF (1988) Interactive influence of infectious disease and genetic diversity in natural populations. *Trends in Ecology and Evolution* **3**, 254-9.

O'Brien SJ, Roelke ME, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M & Wildt DE (1985) Genetic basis for species vulnerability in the cheetah. *Science* **227**, 1428-34.

Ohta T (1991) Role of diversifying selection and gene conversion in evolution of major histocompatibility complex loci. *Proceedings of the National Academy of Science of the United States of America* **88**, 6716-20.

Oliver MK & Piertney SB (2012) Selection maintains MHC diversity through a natural population bottleneck. *Molecular Biology and Evolution* **29**, 1713-20.

Oliver MK, Telfer S & Piertney SB (2009) Major histocompatibility complex (MHC) heterozygote superiority to natural multi-parasite infections in the water vole (*Arvicola terrestris*). *Proceedings of the Royal Society B: Biological Sciences* **276**, 1119-28.

Oppelt C, Starkloff A, Rausch P, von Holst D & Rödel HG (2010) Major histocompatibility complex variation and age-specific endoparasite load in subadult European rabbits. *Molecular Ecology* **19**, 4155-67.

Orr K, Danks A & Gillen K (1995) Two Peoples Bay Nature Reserve : management plan. (ed. by Management DoCaL), Perth, Western Australia.

Ortego J, Calabuig G, Cordero PJ & Aparicio JM (2007a) Egg production and individual genetic diversity in lesser kestrels. *Molecular Ecology* **16**, 2383-92.

Ortego J, Cordero PJ, Aparicio JM & Calabuig G (2007b) No relationship between individual genetic diversity and prevalence of avian malaria in a migratory kestrel. *Molecular Ecology* **16**, 4858-66.

Owen JP, Delany ME & Mullens BA (2008) MHC haplotype involvement in avian resistance to an ectoparasite. *Immunogenetics* **60**, 621-31.

Ozaki K, Yamamoto Y & Yamagishi S (2010) Genetic diversity and phylogeny of the endangered Okinawa rail, *Gallirallus okinawae*. *Genes and Genetic Systems* **85**, 55-63.

Pacioni C, Wayne AF & Spencer PBS (2013) Genetic outcomes from the translocations of the critically endangered woylie. *Current Zoology* **59**, 294-310.

Parham P & Ohta T (1996) Population biology of antigen presentation by MHC Class I molecules. *Science* **272**, 67-74.

Pattison M (2008) *Poultry Diseases*, Elsevier Health Sciences.

Peakall R & Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-95.

Peakall R & Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. *Bioinformatics* **28**, 2537-9.

Peery MZ, Kirby R, Reid BN, Stoelting R, Douet-Bëer E, Robinson S, Vásquez-Carrillo C, Pauli JN & Palsbøll PJ (2012) Reliability of genetic bottleneck

tests for detecting recent population declines. *Molecular Ecology* **21**, 3403-18.

Penn DJ (2002a) Major Histocompatibility Complex. In: *Encyclopedia of Life Sciences*. Macmillian Publishers Ltd, Nature Publishing Group.

Penn DJ (2002b) The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology* **108**, 1-21.

Penn DJ, Damjanovich K & Potts WK (2002) MHC heterozygosity confers a selective advantage against multiple-strain infections. *Proceedings of the National Academy of Science of the United States of America* **99**, 11260-4.

Penn DJ & Potts WK (1999) The evolution of mating preferences and major histocompatibility complex genes. *The American Naturalist* **153**, 145-64.

Pereira HM, Navarro LM & Martins IS (2012) Global biodiversity change: the bad, the good, and the unknown. *Annual Review of Environment and Resources* **37**, 25-50.

Pimm SL, Moulton MP & Justice LJ (1994) Bird extinctions in the Central Pacific. *Philosophical Transcripts of the Royal Society London* **344**, 27-33.

Pimm SL, Russell GJ, Gittleman JL & Brooks TM (1995) The future of biodiversity. *Science* **269**, 347-50.

Pizzey G & Knight F (2007) *The field guide to the birds of Australia*, HarperCollins, Pymble NSW.

Primmer CR, Raudsepp T, Chowdhary BP, Pape Moller A & Ellegren H (1997) Low frequency of microsatellites in the avian genome. *Genome Research* **7**, 471-82.

Pritchard JK, Stephens M & Donnelly O (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-59.

Pruett-Jones S & O'Donnell E (2004) Land birds of Barrow Island: status, population estimates, and responses to an oil-field development. *Journal of the Royal Society of Western Australia* **87**, 101-8.

Pruett-Jones S & Tarvin KA (2001) Aspects of the ecology and behaviour of white-winged fairy-wrens on Barrow Island. *Emu* **101**, 73-8.

Pudovkin AI, Zaykin DV & Hedgecock D (1996) On the potential for estimating the effective number of breeders from heterozygote-excess in progeny. *Genetics* **144**, 383-7.

Pullin AS (2002) *Conservation Biology*, Cambridge University Press.

Queller DC, Strassmann JE & Hughes CR (1993) Microsatellites and kinship. *Trends in Ecology and Evolution* **8**, 285-8.

Radwan J, Biedrzycka A & Babik W (2010) Does reduced MHC diversity decrease viability of vertebrate populations? *Biological Conservation* **143**, 537-44.

Raisin C, Frantz AC, Kundu S, Greenwood AG, Jones CG, Zuel N & Groombridge JJ (2009) Genetic consequences of intensive conservation management for the Mauritius parakeet. *Conservation Genetics* **13**, 707-15.

Ralls K & Ballou JD (2004) Genetic status and management of California condors. *The Condor* **106**, 215-28.

Ralls K, Ballou JD, Rideout BA & Frankham R (2000) Genetic management of chondrodystrophy in California condors. *Animal Conservation* **3**, 145-53.

Ramstad KM, Pfunder M, Robertson HA, Colbourne RM, Allendorf FW & Daugherty CH (2010) Fourteen microsatellite loci cross-amplify in all five kiwi species (*Apteryx* spp.) and reveal extremely low genetic variation in little spotted kiwi (*A. owenii*). *Conservation Genetics Resources* **2**, 333-6.

Rathburn MK & Montgomerie R (2003) Breeding biology and social structure of white-winged fairy-wrens (*Malurus leucopterus*): comparison between island and mainland subspecies having different plumage phenotypes. *Emu* **103**, 295-306.

Rave EH (1995) Genetic analyses of wild populations of Hawaiian geese using DNA fingerprinting. *The Condor* **97**, 82-90.

Raymond M & Rousset F (1995) GENEPOP (version 1.2): population genetics software for the exact tests and ecumenicism. *Journal of Heredity* **86**, 248-9.

Reed DH (2007) Extinction of island endemics: it is not inbreeding depression. *Animal Conservation* **10**, 145-6.

Reppas GP, Hartley WJ & Gill PA (1995) Microfilaraemia in Australian native birds. *Australian Veterinary Journal* **72**, 356-7.

Richardson DS, Jury FL, Dawson DA, Salgueiro P, Komdeur J & Burke T (2000) Fifty Seychelles warbler (*Acrocephalus sechellensis*) microsatellite loci polymorphic in Sylviidae species and their cross-species amplification in other passerine birds. *Molecular Ecology* **9**, 2226-31.

Richardson DS & Westerdahl H (2003) MHC diversity in two Acrocephalus species: the outbred Great reed warbler and the inbred Seychelles warbler. *Molecular Ecology* **12**, 3523-9.

Roache M (2011) The action plan for threatened Australian macropods 2011-2021. WWF-Australia.

Roberts SC, Little AC, Gosling LM, Perrett DI, Carter V, Jones BC, Penton-Voak I & Petrie M (2005) MHC-heterozygosity and human facial attractiveness. *Evolution and Human Behavior* **26**, 213-26.

Rodríguez-Labajos B, Binimelis R & Monterroso I (2009) Multi-level driving forces of biological invasions. *Ecological Economics* **69**, 63-75.

Roelke ME, Martenson JS & O'Brien SJ (1993) The consequences of demographic reduction and genetic depletion in the endangered Florida panther. *Current Biology* **3**, 340-50.

Romanov MN, Tuttle EM, Houck ML, Modi WS, Chemnick LG, Korody ML, Mork EMS, Otten CA, Renner T, Jones KC, Dandekar S, Papp JC, Da Y, NISC Comparative Sequencing Program, Green ED, Magrini V, Hickenbotham

MT, Glasscock J, McGrath S, Mardis ER & Ryder OA (2009) The value of avian genomics to the conservation of wildlife. *BMC Genomics* **10**, S10.

Rosenzweig ML (1995) *Species diversity in space and time*, Cambridge University Press.

Rotheray EL, Greminger MP, Nater A, Krützen M, Goulson D & Bussière LF (2011) Polymorphic microsatellite loci for the endangered pine hoverfly *Blera fallax* (Diptera: Syrphidae). *Conservation Genetics Resources* **4**, 117-20.

Rout TM, Hauser CE & Possingham HP (2007) Minimise long-term loss or maximise short-term gain? Optimal translocation strategies for threatened species. *Ecological Modelling* **201**, 67-74.

Rozen S & Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics methods and protocols: methods in molecular biology* (ed. by S KSM), pp. 365-86. Humana Press, Totowa.

Ruffell J & Parsons S (2010) Translocation of lesser short-tailed bats to Kapiti Island, New Zealand: release site suitability and post-release monitoring. In: *Global Re-introduction Perspectives: Additional case-studies from around the globe* (ed. by P Soorae), pp. 193-7, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu-Dhabi.

Rutkowski R, Keller M & Jagołkowska P (2012) Population genetics of the hazel hen *Bonasa bonasia* in Poland assessed with non-invasive samples. *Central European Journal of Biology* **7**, 759-75.

Saarinen EV & Austin JD (2010) When technology meets conservation: increased microsatellite marker production using 454 genome sequencing on the endangered Okaloosa darter (*Etheostoma okaloosae*). *Journal of Heredity* **101**, 784-8.

Salmona J, Salamolard M, Fouillot D, Ghestemme T, Larose J, Centon JF, Sousa V, Dawson DA, Thebaud C & Chikhi L (2012) Signature of a pre-human population decline in the critically endangered Reunion Island endemic forest bird *Coracina newtoni*. *PLOS One* **7**, e43524.

Sarrazin F & Barbault R (1996) Reintroduction: challenges and lessons for basic ecology. *Trends in Ecology and Evolution* **11**, 474-8.

Sato A, Tichy H, Grant PR, Grant BR, Sato T & O'hUigin C (2010) Spectrum of MHC Class II variability in Darwin's finches and their close relatives. *Molecular Biology and Evolution* **28**, 1943-56.

Sawyer SA (1999) GENECONV: A computer package for the statistical detection of gene conversion. Department of Mathematics, Washington University in St. Louis http://www.math.wustl.edu/~sawyer.

Schröder NWJ & Schumann RR (2005) Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infectious Diseases* **5**, 156-64.

Seddon JM, Parker HG, Ostrander EA & Ellegren H (2005) SNPs in ecological and conservation studies: a test in the Scandinavian wolf population. *Molecular Ecology* **14**, 503-11.

Selander RK (1965) On mating systems and sexual selection. *The American Naturalist* **99**, 129-41.

Sgrò CM, Lowe AJ & Hoffmann AA (2011) Building evolutionary resilience for conserving biodiversity under climate change. *Evolutionary Applications* **4**, 326-37.

She JX, Boehme SA, Wang TW, Bonhomme F & Wakeland EK (1991) Amplification of major histocompatibility complex Class II gene diversity by intraexonic recombination. *Proceedings of the National Academy of Science of the United States of America* **88**, 453-7.

Shendure J & Ji H (2008) Next-generation DNA sequencing. *Nature Biotechnology* **26**, 1135-45.

Sherley GH, Stringer IAN & Parrish GR (2010) Summary of native bat, reptile, amphibian and terrestrial invertebrate translocations in New Zealand. In:

Science for Conservation 303, p. 39. Department of Conservation, Wellington.

Silver R, Andrews H & Ball GF (1985) Parental care in an ecological perspective: a quantitative analysis of avian subfamilies. *American Zoologist* **25**, 823-40.

Simkins G (2008) Re-introduction of Arabian oryx into the Dubai Desert Conservation Reserve, Dubai, UAE In: *Global Re-introduction Perspectives: Re-introduction case-studies from around the globe* (ed. by P Soorae), pp. 188-91, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Smith FDM, May RM, Pellew R, Johnson TH & Walter KR (1993) How much do we know about current extinction rate? *Trends in Ecology and Evolution* **8**, 375-8.

Smith GT (1985) Population and habitat selection of the noisy scrub-bird, *Atrichornis clamosus*, 1962-83. *Australian Wildlife Research* **12**, 479-85.

Smith GT & Forrester RI (1981) The status of the noisy scrub-bird *Atrichornis clamosus*. *Biological Conservation* **19**, 239-54.

Smith GT & Robinson FN (1976) The noisy scrub-bird: An interim report. *Emu* **76**, 37-42.

Smith S, Belov K & Hughes J (2010a) MHC screening for marsupial conservation: extremely low levels of Class II diversity indicate population vulnerability for an endangered Australian marsupial. *Conservation Genetics* **11**, 269-78.

Smith S, Mang T, de Bellocq JG, Schaschl H, Zeitlhofer C, Hackländer K & Suchentrunk F (2010b) Homozygosity at a Class II MHC locus depresses female reproductive ability in European brown hares. *Molecular Ecology* **19**, 4131-43.

Soulé ME (1980) *Conservation biology: an evolutionary-ecological perspective*, Sinauer Associates.

Spangenberg JH (2007) Biodiversity pressure and the driving forces behind. *Ecological Economics* **61**, 146-58.

Spencer CC, Neigel JE & Leberg PL (2000) Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Molecular Ecology* **9**, 1517-28.

Spielmann D, Brook BW, Briscoe DA & Frankham R (2004) Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics* **5**, 439-48.

Spurgin LG, van Oosterhout C, Illera JC, Bridgett S, Gharbi K, Emerson BC & DS R. (2011) Gene conversion rapidly generates major histocompatibility complex diversity in recently founded bird populations. *Molecular Ecology* **20**, 5213-25.

Stiver JR, Apa AD, Remington TE & Gibson RM (2008) Polygyny and female breeding failure reduce effective population size in the lekking Gunnison sage-grouse. *Biological Conservation* **141**, 472-81.

Steiner CC, Putnam AS, Hoeck PEA & Ryder OA (2013) Conservation Genomics of Threatened Animal Species. *Annual Review of Animal Biosciences* **1**, 261-281.

Stockwell CA, Mulvey M & Vinyard GL (1996) Translocations and the preservation of allelic diversity. *Conservation Biology* **10**, 1133-41.

Strandh M, Westerdahl H, Pontarp M, Canbäck B, Dubois M-P, Christian M, Taberlet P & Bonadonna F (2012) Major histocompatibility complex Class II compatibility, but not Class I, predicts mate choice in a bird with highly developed olfaction. *Proceedings of the Royal Society B: Biological Sciences* **279**, 4457-63.

Sutton JT, Nakagawa S, Robertson BC & Jamieson IG (2011) Disentangling the roles of natural selection and genetic drift in shaping variation at MHC immunity genes. *Molecular Ecology* **20**, 4408-20.

Swinnerton KJ, Groombridge JJ, Jones CG, Burn RW & Mungroo Y (2004) Inbreeding depression and founder diversity among captive and free-living populations of the endangered pink pigeon *Columba mayeri*. *Animal Conservation* **7**, 353-64.

Tallmon DA, Luikart G & Waples RS (2004) The alluring simplicity and complex reality of genetic rescue. *Trends in Ecology and Evolution* **19**, 489-96.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-9.

Tarr CL, Conant S & Fleischer RC (1998) Founder events and variation at microsatellite loci in an insular passerine bird, the Laysan finch (*Telespiza cantans*). *Molecular Ecology* **7**, 719-31.

Tarr CL & Fleischer RC (1999) Population boundaries and genetic diversity in the endangered Mariana crow (*Corvus kubaryi*). *Molecular Ecology* **8**, 941-9.

Taylor SS, Jamieson IG & Wallis GP (2007) Historic and contemporary levels of genetic variation in two New Zealand passerines with different histories of decline *Journal of Evolutionary Biology* **20**, 2035-47.

Taylor SS & Jamieson IG (2008) No evidence for loss of genetic variation following sequential translocations in extant populations of a genetically depauperate species. *Molecular Ecology* **17**, 545-56.

Templeton AR, Hemmer H, Mace G, Seal US, Shields WM & Woodruff DS (1986) Local adaptation, coadaptation, and population boundaries. *Zoo Biology* **5**, 115-25.

Thomas ML, Harger JH, Wagener DK, Rabin BS & Gill TJ (1985) HLA sharing and spontaneous abortion in humans. *American Journal of Obstetrics and Gynecology* **151**, 1053-8.

Thornhill R, Gangestad SW, Miller R, Scheyd G, McCollough JK & Franklin M (2003) Major histocompatibility complex genes, symmetry, and body scent attractiveness in men and women. *Behavioral Ecology* **14**, 668-78.

Tiller C, Comer S & Danks M (2007) Noisy scrub-bird recovery progam & Project Phoenix annual report for the South Coast Threatened Birds Recovery Team, Department of Environment and Conservation, Albany WA.

Tompkins DM, Mitchell RA & Bryant DM (2006) Hybridization increases measures of innate and cell-mediated immunity in an endangered bird species. *Journal of Animal Ecology* **75**, 559-64.

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ & Kuris AM (2003) Introduced species and their missing parasites. *Nature* **421**, 628-30.

Tóth G, Gáspári Z & Jurka J (2000) Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research* **10**, 967-81.

Tracy LN, Wallis GP, Efford MG & Jamieson IG (2011) Preserving genetic diversity in threatened species reintroductions: how many individuals should be released? *Animal Conservation* **14**, 439-46.

Tufto J (2001) Effects of releasing maladapted individuals: a demographic-evolutionary model. *The American Naturalist* **158**, 331-40.

Ujvari B & Belov K (2011) Major Histocompatibility Complex (MHC) markers in conservation biology. *International Journal of Molecular Sciences* **12**, 5168-86.

Väli Ü, Einarsson A, Waits L & Ellegren H (2008) To what extent do microsatellite markers reflect genome-wide genetic diversity in natural populations? *Molecular Ecology* **17**, 3808-17.

van Oosterhout C, Hutchinson WF, Wills DPM & Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535-8.

Veillet A, Shrestha R & Price DK (2008) Polymorphic microsatellites in nene, the endangered Hawaiian goose (*Branta sandvicensis*). *Molecular Ecology Resources* **8**, 1158-60.

Vitousek PM (1988) Diversity and Biological Invasions of Oceanic Islands. In: *Biodiversity* (ed. by Wilson E), pp. 181-92, National Academy of Sciences/Smithsonian Institution.

Wang B, Ekblom R, Castoe TA, Jones EP, Kozma R, Bongcam-Rudloff E, Pollock DD & Höglund J (2012) Transcriptome sequencing of black grouse (*Tetrao tetrix*) for immune gene discovery and microsatellite development. *Open Biology*.

Wang J, Caballero A & Hill WG (1998) The effect of linkage disequilibrium and deviation from Hardy-Weinberg proportions on the changes in genetic variance with bottlenecking. *Heredity* **81**, 174-86.

Waples RS & Do C (2010) Linkage disequilibrium estimates of contemporary *Ne* using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. *Evolutionary Applications* **3**, 244-62.

Wayne RK, Modi WS & O'Brien SJ (1986) Morphological variability and asymmetry in the cheetah (*Acinonyx jubatus*), a genetically uniform species. *Evolution* **40**, 78-85.

Weber DS, Stewart BS, Schienman J & Lehman N (2004) Major histocompatibility complex variation at three Class II loci in the northern elephant seal *Molecular Ecology* **13**, 711-718.

Weckerly FW (1998) Sexual-Size Dimorphism: Influence of Mass and Mating Systems in the Most Dimorphic Mammals. *Journal of Mammology* **79**, 33-52.

Weir BS & Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358-70.

Westerdahl H (2007) Passerine MHC: genetic variation and disease resistance in the wild. *Journal of Ornithology* **148**, S469-S77.

Westerdahl H, Wittzell H & von Schantz T (2000) MHC diversity in two passerine birds: no evidence for a minimal essential MHC. *Immunogenetics* **52**, 92-100.

Whitehouse DB (2009) Genes and Genomes In: *Molecular Biology and Biotechnology* (ed. by JM Walker & R Rapley), Royal Society of Chemistry.

Whiteman NK, Matson KD, Bollmer JL & Parker PG (2006) Disease ecology in the Galápagos hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies. *Proceedings of the Royal Society B: Biological Sciences* **273**, 797-804.

Whitwell SM, Amiot C, Mclean IG, Lovegrove TG, Armstrong DP, Brunton DH & Ji WH (2012) Losing anti-predatory behaviour: A cost of translocation. *Austral Ecology* **37**, 413-8.

Wiens JA (1995) Habitat fragmentation: island v landscape perspectives on bird conservation. *Ibis* **137**, S97-S104.

Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, van Dyck A, Ebedes H & Brand DJ (1983) Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. *Biology of Reproduction* **29**, 1019-25.

Wilson EO (1984) *Biophilia*, Harvard University Press.

Wooller RD & Bradley JS (1981) Consistent individuality in the calls of spinifexbirds *Eremiornis carteri* on Barrow Island, WA. 40.

World Conservation Monitoring Centre (1992) *Global biodiversity: status of the earth's living resources.*, Chapman and Hall, London.

Worley K, Collet J, Spurgin LG, Cornwallis C, Pizzari T & Richardson DS (2010) MHC heterozygosity and survival in red junglefowl. *Molecular Ecology* **19**, 3064-75.

Wright S (1931) Evolution in Mendelian populations. *Genetics* **16**, 97-159.

Wright S (1938) Size of population and breeding structure in relation to evolution. *Science* **87**, 430-1.

Zafar-ul Islam M, Boug A, Ismail K, Robinson ER & bin Saleh al Hafedh Y (2010) Over 20 years of re-introducing the Arabian oryx in fenced and free-range protected areas of Saudi Arabia In: *Global Re-introduction Perspectives: 2010 - Additional case-studies from around the globe* (ed. by P Soorae), pp. 269-73, IUCN/SSC Re-introduction Specialist Group & Environment Agency, Abu Dhabi.

Zagalska-Neubauer M, Babik W, Stuglik M, Gustafsson L, Cichoń M & Radwan J (2010) 454 sequencing reveals extreme complexity of the Class II Major Histocompatibility Complex in the collared flycatcher. *BMC Evolutionary Biology* **10**, 395.

Zhang B, Fang S-G & Xi Y-M (2006) Major histocompatibility complex variation in the endangered crested ibis *Nipponia nippon* and implications for reintroduction. *Biochemical Genetics* **44**, 113-23.

Zwifel D, Hoop R, Sachse K, Pospischil A & Borel N (2009) Prevalence of *Chlamydophila psittaci* in wild birds-potential risk of domestic poultry, pet birds, and public health? *European Journal of Wildlife Research* **55**, 575-81.

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.