The Major Histocompatibility Complex (MHC) of the Kiwi (Apteryx spp.).

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Erratum

p iii line 19 The sentence starting "A result.." should read "The result in the rowi sample is more consistent with a remnant population."

p 15 line 15 "it's" should be "its"

p 23 line 25 The sentence starting " While a study..." should read "A study on the inbred human Hutterite population showed an increased foetal loss rate for couples with 16 matching loci"

p 25 line 22 "maybe" should be "may be"

p 28 line 10 "is" should be "are"

p 29 line 5 "Salmonella" should be "*Salmonella*"

p 29 line 23 "adapts" should be "adapt"

p 30 line 19 The sentence starting " Although Hoelzel..." should read " A study by Hoelzel et al (Hoelzel et al 1999), showed a high level of diversity in the Southern Elephant seal, not a low level in the MHC."

p 35 line 24 insert a comma after "individual"

p 36 line 1 "it's" should be "its"

p 40 line 9 insert "is" after "now"

p 47 line 20 "it's" should be "its"

p 54 3.1.1. "as it is" should be "as they are"

p 55 line 25 The sentence starting "Remove as much..." should read " Ethanol was removed, and the precipitate resuspended in ~30µl of milliQ water and stored at 4° C overnight."

p 56 line 22 "were" should be "was"

p 58 line 3 "was" should be "were"

p 67 line 28 "is" should be "was"

p 71 line 31 "is" should be "was"

p 77 line 1 "nomenculture" should be "nomenclature"

p 79 line 3 "was" should be "were"

p 79 line 13 "was" should be "were"

p 79 line 19 "was" should be "were"

p 84 line 18 "locus" should be "loci"

p 84 line 21 The sentence starting "It is hoped..." should read "It will require more work on the kiwi genome to elucidate the organisation of MHC class I and II."

p 84 line 26 "artefacts" should be "artefact"

p 84 line 29 "loci" should be "locus"

p 86 line 23 "encode" should be "encodes"

p 90 line 14 "epidemics" should be "epizootics" repeats

p 90 line 14 "lead" should be "led"

missing bits

p 96 line 16 The sentence starting "These were ..." should read" The guidelines were: to find the earliest division of the NJ tree where any given bird did not have more than two alleles at a given loci and to do this with minimum the number of loci."

p 96 line 26 The sentence starting "Interestingly the ..." should read " Figure 5.4 shows a NJ tree with the most basal division between the two sizes of loci, i.e. 281bp and 284bp."

p 98 – 100 In Figures 5.2, 5.3, and 5.4: the numbers across the top refer to the samples from different birds in that species. The boxes with ticks indicate in that bird (sample) the loci was present.

p 102 line 15 remove "are"

p 102 line 18 "is" should be "are"

p 110 line 11 "loci" should be "locus"

p 121 line 21 "... and the sample size of thie study may not be enough to allow one to readily make an inference about the mode of evolution." should be added to the end of the sentence.

p 123 line 27 The sentence starting "Although the ..." should read" Figures 5.5 and 5.6 show the basal split in the kiwi alleles tends to be between 264bp and 267bp, but this is not absolute."

p 125 line 16 "it's" should be "its"

p 126 line 16 "Westerdahl" should be "Westerdahl's"

p 129 line 23 "principals" should be "principles"

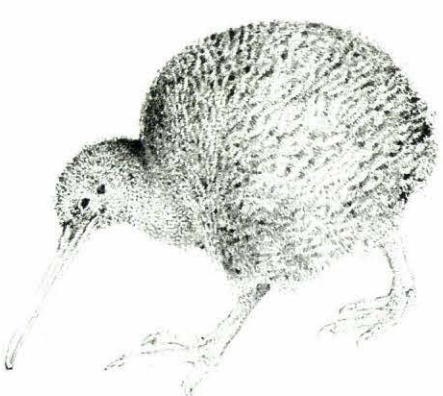
p 131 line 31 "populations" should be "population's"

p 175 line 23 Simkova et al should be on p 183

p 185 line 26 van Oosterhout et al should include the title "Balancing selection, random genetic drift, and genetic variation at the major histocompatibility complex in two wild populations of guppies (poecilia reticulata)"

Rowi (*Apteryx rowi*)

North Island Brown Kiwi (*Apteryx mantelli*)



Little Spotted Kiwi (*Apteryx owenii*)

drawn by Vivian Ward.

Abstract

This thesis investigates the polymorphism of the Major Histocompatibility Complex (MHC) in the threatened New Zealand Kiwi (*Apteryx spp.*). The MHC genes are usually highly polymorphic and play a direct role in disease resistance. A lack of MHC polymorphism may affect the ability of a population to respond to continuously evolving pathogens. The Kiwi is a unique bird, endemic to New Zealand, but despite being considered taonga (a treasure) all five kiwi species are threatened and require active management to sustain current population levels. The role of infectious diseases in the kiwi's past and future survival is currently only a matter of conjecture. To analyse the kiwi MHC and its polymorphism, a PCR and primers were designed that amplified the MHC Class II B exon 2, a protein binding region (PBR) and a site where polymorphism is expected. Feather samples from three different kiwi species, the North Island Brown (*Apteryx mantelli*), the Little Spotted Kiwi (*Apteryx owenii)*, and the Rowi (*Apteryx rowi)* were used as a non-invasive source of DNA. The MHC results for eight Little Spotted Kiwi from Red Mercury Island showed almost no variation in the form of different alleles between birds. Four putative alleles were shared by all birds, each bird having some or all of the alleles. Rowi are only found in Okarito and are a small population of 250 birds. The 18 birds tested showed a greater range of diversity than expected from a bottled necked population with 14 putative alleles and three pseudogenes. A result more consistent with a remnant population. The twelve North Island Brown birds showed a range of polymorphism: 11 putative alleles and two pseudogenes. Analysis of the Kiwi MHC supports the suggestion that avian MHC sequences evolved by concerted evolution and genetic conversion.

Acknowledgements

This work has indeed been accomplished by "standing on the shoulders of giants". I wish to express my sincere gratitude to the many people without whom this project would not be possible.

I am indebted to my mother, Noela Binney, for her help and support throughout my life. Sadly this thesis project began with helping my mother fight one cancer and ended helping her fight another cancer. The support we got, especially from the Whangaporoa Hospice staff and volunteers, for my mother and myself while I was caregiver and simultaneously finishing this thesis was precious to both of us.

Distinguished Professor David Lambert, for the wonderful opportunity and all support he gave me to do this project. Dr Leon Huynen, without whose skill and expertise I might still be in the Lab chasing down dead ends. Lara Shepherd was generous with her maps and work on ancient kiwi DNA. Vivian Ward was generous with her help on making my diagrams better and wonderful kiwi illustrations. Dr Jennie Yanamura was also helpful and supportive throughout.

I also have a special thanks to my lab buddies at the Allan Wilson Centre who helped me on a myriad of levels, Andrew Dodd, Tamara Sirey, Betty Adams, Jarod Young, and Jennifer Anderson, Charlie Gao, and Isabella Cheung for their help in sequencing

Many thanks to the dedicated kiwi wranglers at Rainbow Springs lead by Claire Travis and at Otorohanga lead by Eric Fox and the staff at the Auckland Zoo Kiwi house and Veterinarians Richard Jacob-Hoff and John Potter.

The DOC staff that helped collect samples including: Chrissy Wicks and her team at Franz Josef looking after the Rowi. Kelly Stevens for her help with the DOC paper work. The Te Runanga o Makaawhio iwi for their support that allowed access to Rowi feather samples. Dr Karen Nutt who while at Auckland University collaborated on access to Rowi samples.

Preface

The research undertaken for this thesis had contributions from other researchers.

Sample Collection.

The feather samples were collected by DOC staff and Dr Karen Nutt currently at Waikato University. The samples are from a population of North Island Brown kiwi (NIB) near Whangarei, and a population of Little Spotted Kiwi (LSK) on Red Mercury Island and Rowi a Brown Kiwi species found at Okarito.

The blood sample used for cDNA was collected by the veterinary staff at Auckland Zoo during a routine examination of a resident bird. I performed the rest of the processing of this sample.

DNA extraction.

Dr Karen Nutt and her staff extracted DNA from some of the rowi samples and I extracted the rest. I extracted the DNA from the NIB and LSK feather.

Primer Design.

Initially seven primers designed and published by other researchers were used; they are acknowledged and listed in Table 3.1. Dr Leon Huynen designed two primers, chMHCIIex1F and chMHCIIex3R, this is acknowledged in Table 3.2. The remaining primers I designed, either by eye or using Prime3 online software.

MHC PCR, Cloning, Sequencing and Data Analysis.

I performed the PCR's on the Kiwi DNA, cloned, performed the sequencing reaction and analysed the resulting data. The sequencing with the ABI 3730 Genetic Analyzer was mainly performed by Isabella Cheung. The selection factor identifying the degree of selection on the exon 2 site was analysed with MEGA 4 software with the help of Dr Sankar Subramanian.

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List of Abbreviations used.

A	Adenine
APC	Antigen Presenting Cell.
C	Cytosine
cDNA	complementary DNA (cDNA)
DNA	Deoxyribose Nucleic Acid
DOC	The New Zealand Department of Conservation.
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate.
dGTP	Deoxyguanosine triphosphate
dNTP	A generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP.
dTTP	Deoxythymidine triphosphate.
G	Guanine.
HLA	Human Leukocyte Antigen.
Is	Island
mRNA	messenger Ribose Nucleic Acid
MHC	Major Histocompatibility Complex genes
NZ	New Zealand
PBR	Protein Binding Region
PCR	Polymerase Chain Reaction
RNA	Ribose Nucleic Acid
T	Thymine

Thesis Structure and Format.

Chapter 1 discusses the Major Histocompatibility Complex (MHC) which is important to the adaptive immune system for the identification of self from non-self. This chapter examines not only the structure and function of MHC but the importance of MHC polymorphism, and its role in combating disease. Later in Chapter 5, the relationship of MHC polymorphism to conservation of endangered species like the Kiwi is discussed.

Chapter 2 examines the endangered New Zealand Kiwi (*Apteryx spp.*). In particular, emphasis is given to diseases of the kiwi and how disease can impact on conservation of the kiwi and other species.

Details of the methods and materials used at each stage are reported separately in Chapter 3.

Chapter 4 explains how the project progressed from starting with non specific degenerate primers to finally developing a specific pair of primers for kiwi MHC. A flowchart is present to outline the stages involved. The steps taken to reduce the generation of laboratory artefacts at each stage are also discussed.

Chapter 5 shows the amount of MHC polymorphism found in three different populations of kiwi and compares the results to those of other avian and mammalian MHC.

Chapter 6 summarises the results found in this thesis and their implications for future research.

The Appendixes contain various data to support the thesis.

Chapter 1

The Major Histocompatibility Complex (MHC): MHC Polymorphism.

" Information consists of differences that make a difference": Edward Tufte

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Overview.

This chapter discusses the Major Histocompatibility Complex (MHC) genes, which are thought to be important for survival of the individual and conservation of endangered populations. The MHC plays a pivotal role in the recognition of self from non-self for the immune system. The structure and functions of MHC will be reviewed with emphasis on avian MHC. The MHC contains sections that are conserved throughout vertebrate evolution and other areas that are dynamically changing and accumulating variety i.e., polymorphism, which is attributed to balancing selection. Balancing selection is believed to maintain MHC polymorphism but other features associated with MHC such as mate choice and maternal-foetal interactions have been implicated. Most populations show high levels of polymorphism at classical MHC sites which is thought to enable a flexibility of response to endemic and emerging infectious diseases. However endangered species have by definition small populations and this can result in a loss of MHC polymorphism and may increase the risk of infectious disease epidemic substantially contributing to their extinction.

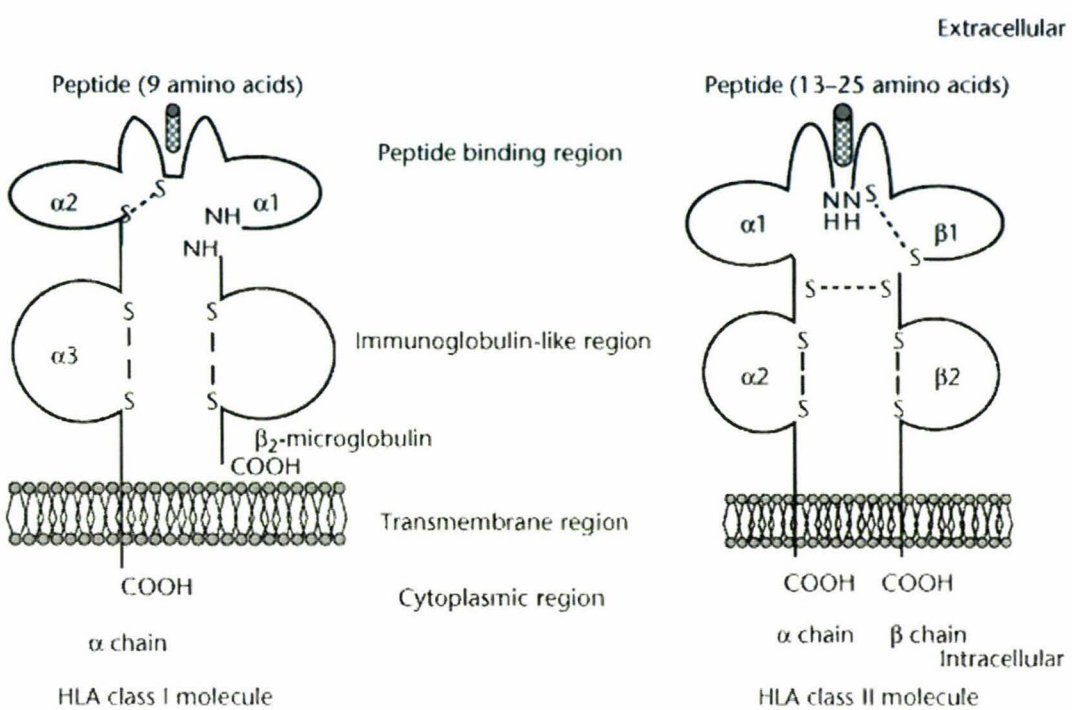
The Structure of MHC

The MHC is a multigene grouping of an immunoglobin superfamily, and pivotal to the functioning of the adaptive immune system. The genomic organisation and action of the MHC can be traced through vertebrates almost to their beginning; it has been found in all jawed vertebrates examined but not in Agnatha (early jawless vertebrates)such as hagfish and lampreys (Flajnik et al 1999).

Classical and non-classical MHC.

The genes within the MHC are often divided into two : "classical" referring to those that are highly polymorphic and highly expressed e.g., class I, class II & class III genes, while "non–classical" genes are not as polymorphic nor as highly expressed (Arnaiz-Villena et al 1997). The extreme levels of polymorphism in classical MHC DNA sequences (Hedrick 1994; Hughes and Hughes 1995) are non-random in distribution and concentrated in the "Protein Binding Region" (PBR) of Class I & II (Hughes and Nei 1988). This indicates that different evolutionary forces act in different regions of the MHC (Cereb et al 1997). The two major subfamilies in the classical MHC are Class I and Class II which have similar form and function and are believed to have a common evolutionary root (Madden 1995). See Figure 1.1 for the glycoprotein structures of class I and class II MHC that are involved in the processing and presentation of self and non-self peptides to T cells. Both are heterodimers expressed on the cell membrane with a large extracellular segment, a transmembrane region and a cytostolic tail. Figure 1.2 is a diagram of the relationship of the protein and it's encoding DNA structure of Class II MHC.

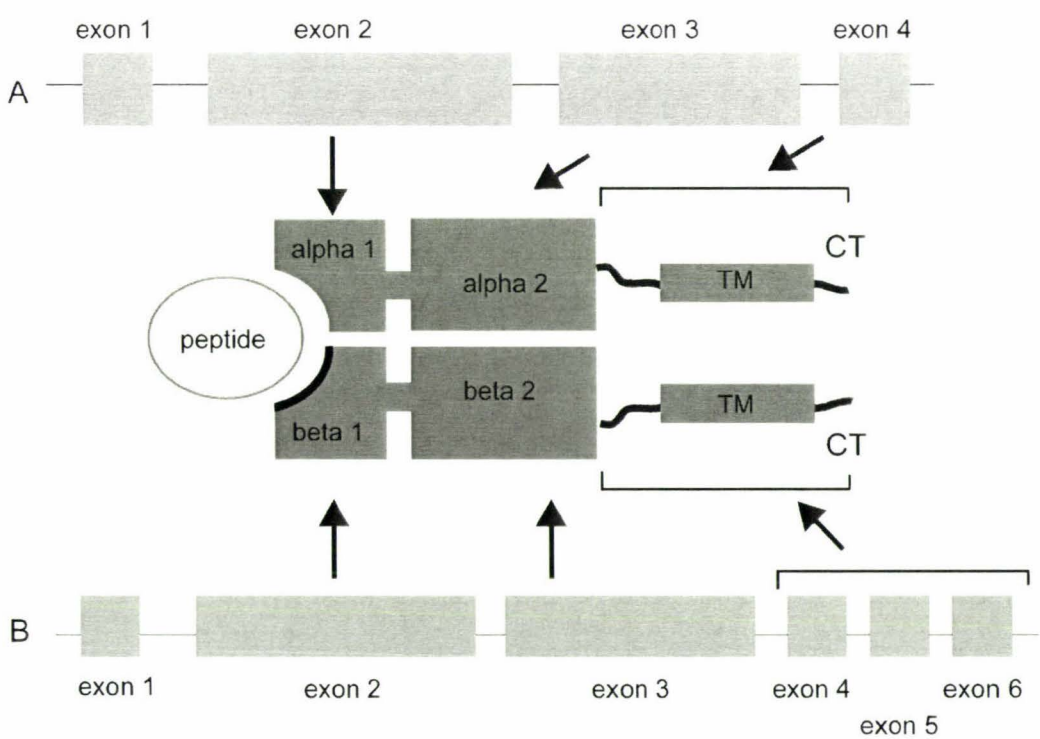
Figure 1.1 The generalized structure of the MHC class I and class II heterodimer glycoproteins. (based on HLA i.e., human MHC)



This graphic is adapted from the Encyclopaedia of Life Sciences published by John Wiley & Sons, Ltd.

Figure 1.2 shows the Class II MHC is a heterodimer glycoprotein formed by an Alpha (A or α) chain protein, coded for by a Class II A DNA sequence, and a Beta (B or β) chain protein, coded for a Class II B DNA sequence. A Protein Binding Region (PBR) is formed by a fold made by the alpha 1 (α1) section of the A chain and the beta 1 (β1)section of the B chain. After some intracellular processing the heterodimer protrudes through the cell membrane with the PBR on the exterior surface of the cell, ready to present a small peptide to a T cell. The heterodimer is anchored to the cell by a transmembrane region (TM) which passes through the cell membrane into the cell and a cytoplasmic tail region (CT) that hangs in the cytoplasm. Exon 1 codes for a leader protein that is discarded during the processing the Class II protein into a heterodimer. The research in this thesis is based on the polymorphism of the Class II B exon 2 of the kiwi (*Apteryx spp.*).

Figure 1.2 Diagram of the DNA and resulting peptide structure of Class II MHC.



(TM = transmembrane region, CT = cytoplasm tail) Diagram of the peptide structure and intron-exon organisation of Class II MHC. Modified from "Molecular methods in Ecology" Ed. Allan J Baker (Baker and Parkin 2000) with the relative (not to scale) position each exon on the structure.

The entire MHC has been sequenced in humans (HLA) and several other mammals including the dog (*Canis familiaris*) (DLA) (Wagner 2003) and pig (*Sus scrofa*) (SLA) (Chardon et al 2000). To date only two avian species – the chicken (*Gallus gallus*) (Guillemot et al 1989) and the Japanese quail (*Coturnix japonica*)(Shiina et al 2004b) have also had the entire MHC region sequenced. The most studied MHC is the human MHC, called the HLA (Human Leukocyte Antigen). It is a single large sequence, 3.6 Mbp long, containing immune related (approx. 40%) and non immune related genes on chromosome 6 (Sequencing Consortium Mhc 1999). The HLA is an area of high gene density with at least 224 identified genes and contains the largest number of polymorphic proteins in the human genome, e.g., HLA-DRB1 has 549 alleles and HLA-DRB has 633 alleles (Kulski J K and Inoko 2006).

In comparison to mammals, the chicken MHC consists of two sections. The B complex a small 92 kbp, and an even smaller separate fragment that is in linkage disequilibrium called the Rfp-Y. Both complexes reside on the chicken microchromosome No 16 (Fillon et al 1996). The chicken MHC sequence is not only much smaller in size than other known sequences, it has no identified pseudogenes and small introns, leading Kaufman (1999) to describe it as the "minimal essential MHC". The compact nature of the avian MHC has been suggested as a consequence of having avian microchromosomes (Kaufman and Wallny 1996). However research showing a larger quail MHC (156 kb) and the presence of pseudogenes in passerines does not support the concept of "minimal essential MHC" being representative for all avians (Hess et al 2000; Westerdahl et al 2000; Aguilar et al 2005). See Figure 1.3 for a comparison of MHC organisation of humans and birds.

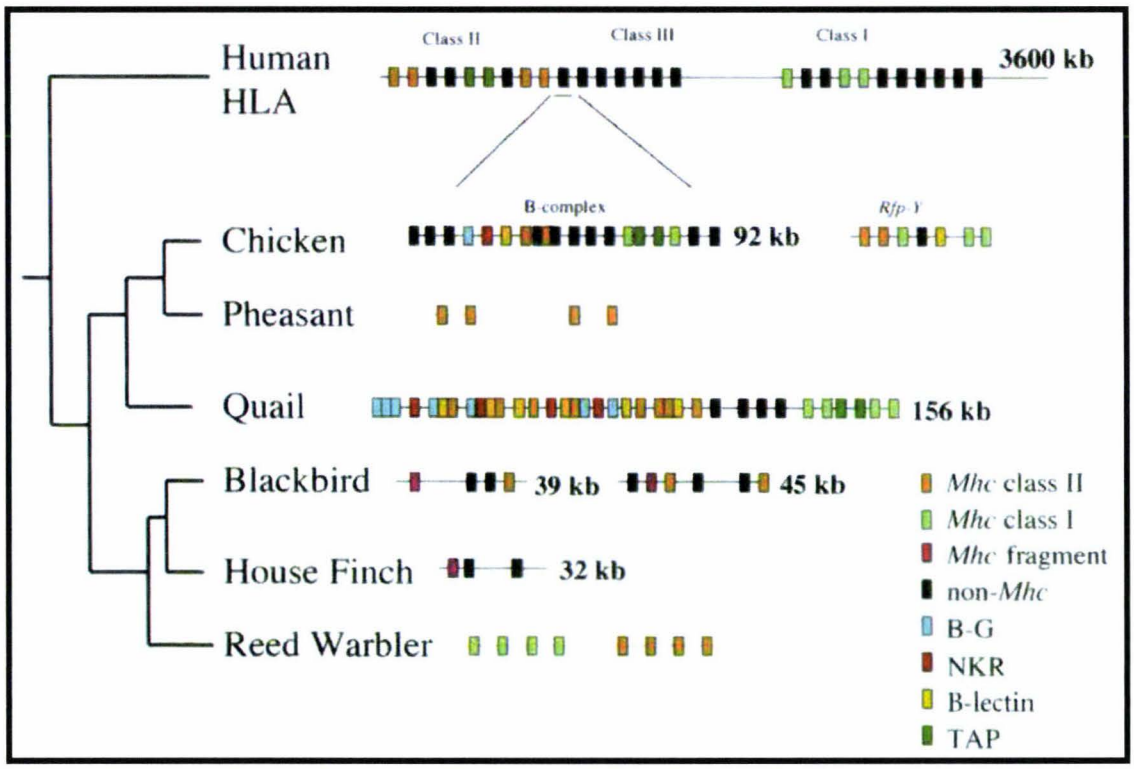


Figure 1.3 Genomic organisation of MHC in birds and humans. This is a simplified representation of the MHC with each box representing more than one gene, except the TAP genes. This diagram is from Hess and Edwards (2002).

Some linkage of MHC genes is apparent in all gnathostomes (jawed vertebrates), but the genomic organization can differ greatly by species, suggesting rapid evolution of MHC genes after divergence from a common ancestor (Kelley et al 2005). The genomic structure of the MHC in birds and mammals has classical Class I & II genes linked together in a large single gene complex (Hughes and Yeager 1998b; Hess and Edwards 2002). It has been speculated that this clustering of genes with related functions provides an advantage by facilitating co-evolution of related genes (Trowsdale 2002). However there is evidence for a group of non-MHC linked Class I genes in the amphibian *Xenopus* and teleost fish with Class I & II genes not localised on the same linkage group (Flajnik et al 1993; Hansen et al 1996; Jasna Bingulac-Popovic et al 1997). The diversity found in the MHC among species is in part due to the losses and gains of various loci. This has been attributed to a compensation for loss of loci in one area by gains of loci in another area (Kelley et al 2005). Examples include the cat (*Felis catus*) which lacks a class II DQ sub region but has an expanded class II DR sub region

(Yuhki et al 2003), or cattle and sheep which have lost the class II DP sub region but compensate with the new clusters of DI/DY genes (Scott et al 1987; Stone and Muggli-Cockett 1990; van der Poel et al 1990; Wright et al 1994).

Generally MHC shows remarkable conservation of organization and function in class I and II genes, even areas that encoded proteins for the polymorphic peptide binding region (PBR) (Madden 1995; Trowsdale 1995). This conservation is not due to DNA being "frozen", since phylogenetic analysis suggests MHC evolves in mammals by a birth-and-death of gene process (Nei et al 1997). Cycles of expansion and contraction of the genes within the MHC are thought to have occurred with a single ancestral gene expanding through serial duplications (births), changing with mutations, and contracting with deletions (deaths) (Klein et al 1993b). Different numbers of genes in different haplotypes, even within the same species or closely related species indicates that gene duplication and decay are on-going. Some researchers consider avian MHC shows more signs of concerted evolution which does not exclude the birth-and-death model as they both may be part of the same process operating over a different time scale (Edwards et al 1999; Wittzell et al 1999).

Functions of the MHC.

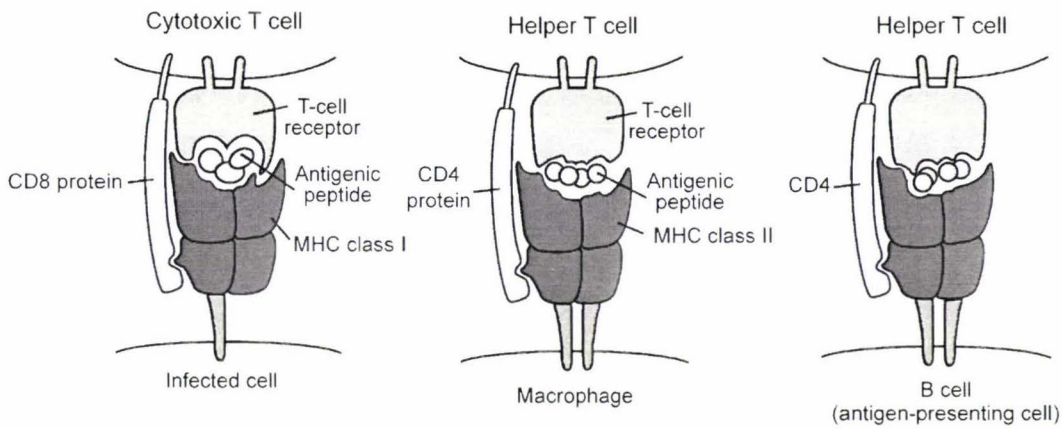
The MHC has been called "the centre of the immune universe" by Trowsdale (Trowsdale 1995), because of its role in differentiating self from non-self. The MHC is part of the adaptive immune system, and just as MHC has not been found in animals earlier than gnathostomes neither has evidence of an adaptive immune system been found (Klein et al 1983; 1995). In some species roles in reproduction like mate choice, kin recognition (Brown and Eklund 1994; Jordan and Bruford; Penn and Potts 1999) and maternal-foetal interactions (Ober 1999) have also been suggested for MHC. All these functions are not mutually exclusive, and could be acting concurrently to maintain MHC polymorphism (Apanius et al 1997).

The Immune function of the MHC.

The Class I genes encode for glycoproteins that act as receptors and are found on all nucleated somatic cells. The Class I receptor on a cell presents a peptide to a T cell receptor on a cytotoxic CD8+ T cell. The peptide being presented is held in a groove or

cleft called the "Protein Binding Region" (PBR) and usually originates from within the cell (endogenous source). Hence Class 1 alleles are more associated with intracellular pathogens such as viruses and acts to identify the infected cell for destruction. Class I receptors can also present a cells own peptides which should not interact with cytotoxic CD8+ T cells to stimulate an immune response. The Class I PBR is a cleft closed at both ends, formed by the ά 1 & ά 2 domains and it fits an 8-10 amino acid long peptide. Figure 1.4 shows the interaction of class I & II MHC receptors with T cell receptors.

Figure 1.4 Major Histocompatibility Complex (MHC) Peptide presentation. This diagram shows Class I & II MHC receptors presenting antigenic peptides to Cytotoxic and Helper T cell receptors.



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Class II genes also encode for glycoproteins that act as receptors, but they are found on Antigen Presenting cells (APC) such as dendritic cells and macrophages. The Class II receptor also interacts with a T cell receptor by presenting a peptide in the "Protein Binding Region" (PBR) cleft but only to cytotoxic CD4+ helper T cells. The origin of peptides held by the class II receptor is often outside of the cell (exogenous source) and hence class II alleles are more associated with extracellular pathogens like many bacteria. However there is evidence that the source is not exclusively exogenous e.g., Class II MHC is important in the immune response to the intracellular pathogen *Mycobacterium tuberculosis* (Torres et al 2006). The PBR cleft is open and formed by

the α1 and β1 domains, and it can hold a longer peptide (13-25 amino acids) than the Class I PBR.

An individual's adaptive immune system can recognise and respond to a limited range of antigens, possibly in part to stop the formation of autoimmune diseases. Peptide–MHC bindings are both stable and promiscuous i.e., although each individual is equipped with only a limited number of different PBR, they can potentially bind to a large array of different peptide antigens and hold them for interaction with T cells (Madden 1995). However there are still limits to the range of peptides each Class I or Class II PBR can hold. The specific size, shape, charge distribution, hydrophilic or hydrophobic nature of the amino acids that form the PBR (the cleft) in the Class I & Class II receptors places a limit on the range of peptides they can "hold" for a T cell receptor. If the size, shape, charge distribution, hydrophilic or hydrophobic nature of the amino acids in the peptide do not align with those of the PBR, it is not "held" or presented to the T cell and hence there is no initiation of the adaptive immune response. There are also limits placed on the range of interactions by the T cells. Early in a mammal's development immature thymocytes which develop into the mature T-cell undergo positive selection for their receptors ability to bind to the bodies own Class I & Class II receptors. The T cell receptors must successfully interact with the Class I & II receptors or the thymocytes are removed by apoptosis. The next stage of thymocyte development involves negative selection to remove those thymocytes with receptors that have too high an affinity for the individuals own MHC when empty or when carrying the individuals own peptides (self-antigen). These self reactive thymocytes are removed by apoptosis, creating self tolerance i.e., selecting against autoimmune diseases.

More MHC Functions.

Other important traits that may be MHC based are mate choice, kin recognition (avoiding inbreeding and aiding social structures) and maternal–foetal interactions e.g., abortions. These functions could all be important, singularly or in combination, to the maintenance of the extreme polymorphisms of the MHC (Apanius et al 1997).

Mating Preferences:

There is evidence for MHC-based mate choice with preference for unrelated individuals (disassortative mating) in several species; mice and man, as well as salmon (for recent reviews see (Grob et al 1998; Jordan and Bruford 1998; Ober 1999; Penn and Potts 1999; Landry et al 2001). However Westerdhal (2004) found no evidence of an MHC-based female mating preference for either genetic compatibility or heterozygosity in the Great Reed Warblers. Alternatively mating choice may be indirectly linked to MHC as suggested by the Hamilton-Zuk hypothesis (1982). Disease resistance may give visual clues in secondary sexual characteristics, which in turn can affect mate choice and this idea is supported by a study in pheasants (Von Schantz et al 1989).

The detection of an individuals MHC haplotype has been attributed to MHC based odour cues (Yamazaki 1976; Yamazaki et al 1983), that may be generated by the odour cue or its precursor being directly bound to some MHC products and then released into serum and concentrated in urine (Yamazaki et al 2000; Carroll et al 2002). Alternative hypotheses are that the MHC based odour could be due to MHC based selection of specific bacteria colonization or co-expression of odour producing genes in the MHC region (Eggert et al 1999). There is evidence in mice and fish of learnt discrimination of similar and dissimilar MHC early in their development from familial imprinting (Beauchamp et al 1988; Penn and Potts 1998b; Olsen et al 2002). Some studies suggest a form of discrimination in humans based on body odour, with women showing a preference for dissimilar HLA body odour (Wedekind and Furi 1997; Santos et al 2005). Great ability in discrimination has been indicated in the female stickleback fish (Gasterosteus aculeatus). The female fish not only prefers the scent of males with high MHC heterozygosity they also "self-reference" their own MHC to provide their offspring with an "optimal set" of MHC alleles (Reusch et al 2001).

Olfaction has not generally been considered an important sense in avian species. However more evidence is accumulating that it is utilised by birds e.g., Blue Petrels (*Halobaena caerulea*) use smell to find their burrows in the dark (Bonadonna et al 2004). The nocturnal kiwi (*Apteryx spp.*) is noted for its large well developed olfactory bulb (Bang and Cobb 1968) with which it discriminates odours for territory marking (Jenkins and Potter 2001) and possibly food hunting (McLennan 1991)(

contra(Cunningham et al 2007)). This makes the kiwi a likely avian candidate to investigate for use of MHC related odours in activities like mate choice and kin recognition.

Kin recognition:

The same MHC properties of high levels of polymorphism and a highly specific marking odour, could act in kin recognition and help avoid inbreeding (Potts and Wakeland 1993; Brown and Eklund 1994; Potts et al 1994; Penn and Potts 1998a). Sibling recognition by MHC odour is supported by studies in fish (Olsen et al 1998; Rajaruna et al 2006) and parent offspring recognition by MHC odour has been shown in mice (Yamazaki et al 2000). Manning et al. (1992) showed female mice had a preference to communally nest with MHC similar females when siblings were unavailable. However Ehman & Scott (Ehman and Scott 2001) could not find an MHC odour preference in congenic female mice.

Maternal–foetal interactions: The paradigm of MHC based maternal–foetal interactions suggests mothers favour more heterozygous MHC off-spring, at various prenatal stages of development. The paradox, as Medawar (1953) pointed out with mammals is the dependence during pregnancy on the foetus and mother retaining immunological tolerance of each other although they are different, when under non-pregnant conditions non-self is rejected.

Maternal selection based on the MHC of offspring may occur, before (specific sperm haplotypes selection), during (selection by the unfertilised egg) and after zygote formation (non implantation and spontaneous abortion) and is not limited to mammalian species (Wedekind 1994). Early work in mice showed MHC involvement in maternal–foetal interactions favours offspring with dissimilar MHC (Clarke & Kirby 1966). While a study on the inbred Hutterite human population showed that there was increased foetal loss rates for couples with 16 matching HLA loci (Ober et al 1998). It was suggested insufficient stimulation of the mother by "foreign" paternal antigens increases the risk of foetal loss. The search to confirm maternal selection of sperm for MHC heterozygosity has suffered from inconclusive evidence of MHC (HLA) expression by human sperm (which may be a developmental regulated expression), and

the female gamete (Haas Jr and Nahhas 1986; Kurpisz et al 1987; Fernandez et al 1999; Martin-Villa et al 1999). However there is evidence of embryo's expressing Class I MHC in mice, the pre and post implantation and the importance of MHC to reproductive success (Fernandez et al 1999). Interestingly a parental viral infection at fertilisation promotes MHC heterozygous embryos compared to non-infected matings (Rulicke et al 1998).

The Polymorphism of the MHC gene.

There is considerable evidence that the extreme amount of MHC polymorphism generally observed emerges from a combination of point mutation, gene conversion and balancing selection (Potts and Slev 1995; Edwards and Hedrick 1998).

Generation and Maintenance of MHC Polymorphism.

Originally the generation and maintenance of the extreme MHC polymorphism was attributed to an increased mutation rate followed by selection for diversity (rapid post speciation diversification)(Klein 1978). Hughes and Nei (1988) dismissed an increased mutation rate at the MHC after examining the pattern of nucleotide substitution between polymorphic alleles in the Class I PBR (Protein Binding Region) region of humans and mice. Estimates of synonymous substitution rates at MHC loci show similar neutral mutation rates within MHC loci to other primate loci, supporting no comparative increased mutation rate in the MHC (Klein et al 1993a; Satta et al 1993). Although work by Edwards et al. (1997) showed that synonymous substitution rates (silent substitution rates) in some MHC class II genes of rodents were as much as four times higher than those measured in primates, the authors suggested that this showed different generation time effects causing variable mutation rates in mammalian MHC .

Gene conversion is a form of recombination where there is non-reciprocal transfer of information (DNA) between homologous sequences. Many MHC alleles differ in short stretches of sequence (patchwork patterns), and occasionally identical short sequence can be found in another allele (Trowsdale 2002). However the exchange of long sequences can promote high similarity of alleles between loci, paradoxically gene conversion may explain both pronounced diversity and extreme similarity in the avian MHC (Hess and Edwards 2002; Westerdahl 2007). See Figure 1.5 for an example of

gene conversion rearranging DNA sequence on a chromosome. The importance of gene conversion to the generation of MHC polymorphism is debated, with some authors identifying the MHC as a "hotspot" of gene conversion by gene micro-recombination and crossovers (reviewed by (Frisse et al 2001; Kauppi et al 2004)). Others consider that as gene conversion events are found throughout the genome it is unlikely to be an important factor in MHC diversity (reviewed by (Zangenberg et al 1995; Martinsohn et al 1999)). The formation of double crossovers between loci can break up existing haplotypes, and by reshuffling the old haplotypes to effectively generate new haplotypes. This results in new combinations and hence more polymorphism in the MHC. Studies in primates and ungulates have shown a concentration of these events at specific loci within the MHC (Gaur and Nepom 1996; Schaschl et al 2006). Recombinations are particularly focused in hypervariable regions like the MHC class II DRB exon 2, which codes for a PBR. Analysis of non mammalian MHC has also shown that in Salmon there are areas of microrecombinations (Langefors et al 2001b) and in avian species there is gene conversion and recombination (Garrigan and Edwards 1999; Edwards et al 2000; Miller and Lambert 2004a; Edwards and Dillon 2005). Avian rates of interlocus gene conversion may be higher than mammalian, as class II sequences cluster within species and orthologous loci have only been identified in closely related species while mammals have had orthologous loci identified in distantly related species (Edwards et al 1995b; Edwards et al 1999; Wittzell et al 1999; Takahashi et al 2000).

Balancing selection

Balancing Selection is a special form of Darwin's natural selection and maybe selecting for the pattern of extreme polymorphism seen in the MHC (reviewed in (Hughes and Nei 1988; Potts and Slev 1995; Hughes and Yeager 1998a; Hedrick 1999; Bernatchez and Landry 2003). The characteristics of MHC polymorphism cannot be accounted for by the mechanisms that operate under neutral evolution (Hughes and Yeager 1998a), but investigations into MHC in some bottlenecked natural populations have found genetic drift, a mechanism of neutral evolution, can still operate strongly on MHC (Miller and Lambert 2004b; Seddon and Ellegren 2004).

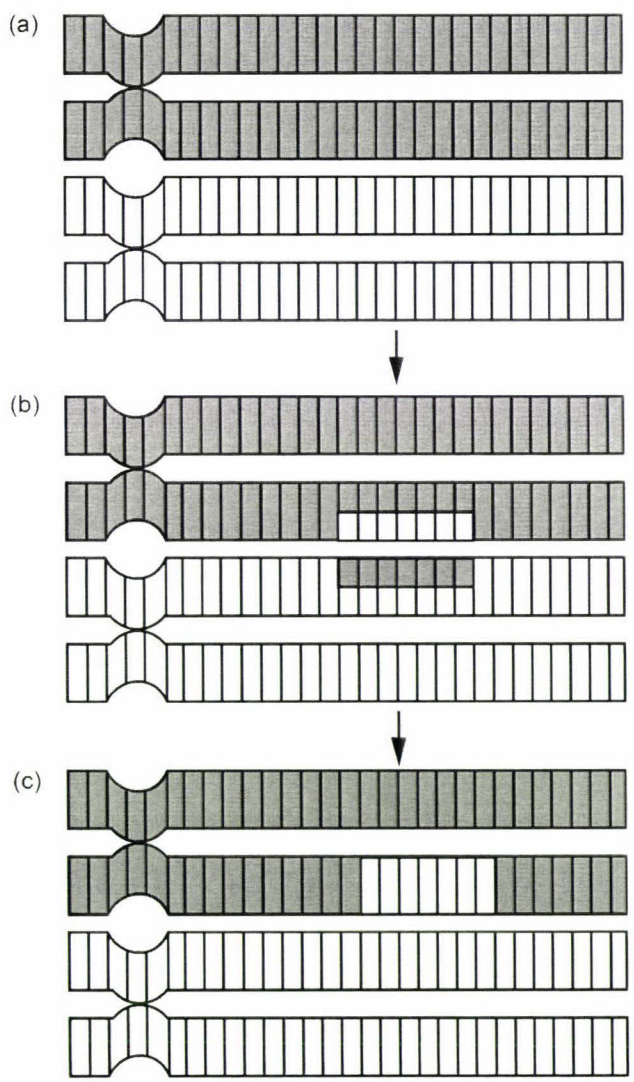
Evidence for Balancing Selection maintaining classical MHC polymorphism is mainly indirect. A possible explanation for the difficulties in directly detecting balancing

selection acting on MHC in a natural population is that selection intensities can vary greatly over time and space, and can depend on the species and experimental context (Edwards and Hedrick 1998). For example, Aguilar (Aguilar et al 2004) estimated a high periodic selection coefficient (s = 0.5) for the San Nicholas Is fox's MHC although most other population studies have recorded much smaller estimates (~s <0.05) (Satta et al 1994; Slatkin and Muirhead 2000; Garrigan and Hedrick 2001; Langefors et al 2001a; 2004).

High number of alleles at uniform allelic frequencies - The allelic frequency distribution of MHC loci shows large numbers of alleles at intermediate frequencies (Potts and Wakeland 1990; Meyer and Thomson 2001). This does not fit the expectation of selective neutrality where equilibrium heterozygosity is a function of genetic drift and mutation rate; neutrally evolving protein coding loci show a pattern of one common allele and one or a small number of other rare alleles (Hedrick and Thomson 1983). Nor does it fit a simple directional (purifying) selection pattern.

High non-synonymous substitution rates at the PBR codons - The peptide-binding region (PBR) codons of both class I and class II MHC molecules, generally show an unusual pattern of nucleotide substitution. At these codons, the number of non-synonymous (amino acid altering) nucleotide substitutions per non-synonymous site greatly exceeds the number of synonymous substitutions per synonymous site i.e., an accumulation over time of mutations that change the amino acid structure of the groove making a functional difference compared to those DNA changes that are silent and do not alter the expressed structure. This pattern, which is the opposite of that seen in most genes, has been taken as evidence of positive Darwinian selection favouring diversity at the amino acid level in the PBR (Hughes and Nei 1988; Hughes and Nei 1989b)

Figure 1.5 Gene conversion is a process by which DNA sequence information is transferred from one DNA helix (which remains unchanged) to another DNA helix, whose sequence is altered. It is shown here occurring in a cell between parental homologous chromosomes in meiotic prophase. After alignment shown in (a), recombination between the inner two chromatids produces a heteroduplex with two mismatches in (b). The latter are repaired by excision and DNA synthesis . This results in (c) where the grey chromatid has undergone a gene conversion from the white chromatid which is unchanged. This diagram is adapted from the Encyclopaedia of Life Sciences Published by John Wiley & Sons, Ltd.



Ancient allelic lineages (also called transpecies evolution or trans-species polymorphism) Some allelic lineages at MHC loci are very ancient, having been maintained for millions of years, predating speciation events especially in mammals (Lawlor et al 1988; Mayer et al 1988; Gyllensten and Erlich 1989). MHC polymorphism is maintained for a much longer time than is expected for a selectively neutral polymorphism which would be expected to either be lost or fixed by genetic drift. The clearest demonstration of this long-lasting polymorphism is seen at loci with low levels of interallelic recombinant rates, as high levels would interfere with retention of the ancient alleles (Hughes et al 1993; McAdam et al 1994; Hughes 2002).

Deficiencies of MHC homozygotes in populations. There is statistically fewer than expected MHC homozygotes in human and semi-natural populations i.e., MHC heterozygotes are much more common in a population than pure chance allows indicating a selection for diversity. (Black and Salzano 1981; Dorak et al 2002).

Linkage disequilibrium among loci. Analysis of MHC has shown high levels of linkage disequilibrium i.e., non-random associations between MHC genes at different loci (Garrigan and Edwards 1999; Edwards and Dillon 2005), supporting the action of a form of selection, but not only balancing selection.

Different forces acting on exons and adjacent introns. When classical polymorphic MHC loci are compared between alleles, the introns are more similar to each other (homogenised) due to recombination and genetic drift, than synonymous exon sites. This implies a different selection force acting on the intron to the force generating polymorphism in the adjacent exon (Cereb et al 1997; Garrigan and Edwards 1999).

Two mechanisms, which are not mutually exclusive, have been proposed for balancing selection and its maintaining of MHC polymorphism, the Heterozygous Advantage hypothesis (overdominant selection) and Frequency-dependent Selection (Jeffery and Bangham 2000; Richman 2000; Penn et al 2002; Bernatchez and Landry 2003). Both heterozygote advantage and frequency-dependent modes of selection rely on forms of rare allele advantage, as rare alleles are most frequently present in heterozygous individuals. (Apanius et al 1997).

Doherty and Zinkernagel (1975) suggested that MHC heterozygotes are able to detect a wider range of pathogens to which an individual may be exposed. This results in heterozygotes having a greater fitness compared to homozygotes. This can be seen in mice when heterozygous individuals survived with greater body weight than homozygotes after exposure to multiple Salmonella strains (Penn et al 2002). Van Oosterhout suggested that heterozygous advantage maintains MHC polymorphism in a small guppy population with high levels of parasitism, despite being exposed to strong genetic drift (van Oosterhout et al 2006). Some have suggested heterozygous advantage can be divided into two categories, (1) 'symmetric overdominance' or 'symmetric balancing selection' (Takahata and Nei 1990) where all heterozygotes are selectively equivalent, and (2) 'divergent allele advantage' where heterozygotes carrying more divergent allelic sequences and hence presenting a broader spectrum of antigens to the immune system have a selective advantage relative to individuals carrying relatively similar alleles and a narrower repertoire (Wakeland et al 1990).

Negative frequency dependent selection occurs when alleles at low frequencies are favoured by selection in comparison to the more common alleles. For example a parasite able to evade detection by the more common alleles has a greater advantage than if it was only able to evade detection by less frequent alleles (Takahata and Nei 1990). Host-parasite dynamics are considered to represent a co-evolutionary "arms race" (Lively and Dybdahl 2000), with a time lag involved which can lead to varying spatiotemporal selection directions in space and time (diversifying selection in space and time) and to a cycling of fitness values of different alleles/genotypes in both hosts and pathogens (Nevo and Beiles 1992; Hedrick 2002; Hedrick 2004) e.g., HIV may adapts to the most frequent alleles in a population, providing a selective advantage for those individuals who express rare alleles. (Trachtenberg et al 2003).

Without incontrovertible evidence for a solely pathogen driven mechanism maintaining MHC's extreme polymorphism some investigators consider that it works in combination with other processes such as disassortative mating which prevents inbreeding and maternal-foetal interactions may be important (Apanius et al 1997; Penn and Potts 1999; Meyer and Thomson 2001). Alternatively Satta (Satta et al 1998) has

suggested MHC polymorphism may be a compromise due to the dual function of MHC to react to non-self epitopes and not to self epitopes. As "self" peptides initiating an immune response would result in autoimmune disease, is at least as disastrous as failure to recognise an invading pathogen (non-self).

MHC Monomorphism.

The classical pattern of high levels of MHC polymorphism has not been found in all populations that have been studied, the four main explanations proposed for the lack of MHC polymorphism found are:

Genetic bottleneck(s) either recently or in the ancient past. This has enabled genetic drift to act on a species with limited range and small declining populations decreasing MHC polymorphism. Such populations often are species that have high conservation value e.g., Asiatic lion (*Panther leo persica*) and the Chatham Is Black Robin (Petrocia traversi) (Yuhki and O'Brien 1990; Miller and Lambert 2004b).

Epidemic diseases that have eliminated MHC diversity by a selective sweep removing haplotypes unable to effectively deal with the pathogen e.g., the common chimpanzee (Pan troglodytes) (de Groot et al 2002).

Low levels of exposure to pathogens, result in a reduction of selection pressure (balancing selection) on a population, possibly due to life style e.g., Southern Elephant seal (*Mirounga leonina)* (Slade 1992). Although Hoelzel (Hoelzel et al 1999) reported high levels of diversity in the Southern Elephant seal .

Combined demographic and lifestyle features such as an obligate monogamous mating system in combination with limited gene flow and limited gene pool due to factors like forest fragmentation e.g., Malagasy giant jumping rat (Hypogeomys antimena).(Sommer et al 2002b) (Sommer 2003).

See Table 1.1 for examples of Wildlife populations with low MHC polymorphism.

Table 1.1 Species with Low Polymorphism at MHC loci and Proposed Causes.

Name	MHC low variance	Proposed Cause	Variation at neutral loci	Reference
Chatham Is. Black Robin *(Petroica traversi)*	Low Class 2	Genetic Bottleneck	Low	(Miller and Lambert 2004b)
Hawaiian honeycreeper *Drepanidinae* spp.	Low Class 2	Genetic Bottleneck	?	(Jarvi et al 2004)
Crested ibis *(Nipponia nippon)*	Low Class 2	Genetic Bottleneck	?	(Zhang et al 2004)
Northern Elephant seal *(Mirounga angustirostris)*	Low Class 2	Genetic Bottleneck	Low	(Hoelzel et al 1999)
Southern Elephant seal *(Mirounga leonina)*	Low Class 2	Exposure to Few Pathogens	moderate	(Slade 1992)*
Hawaiian monk seal *(Monachus schauinslandi)*	Low Class 1	Genetic Bottleneck	?	(Aldridge et al 2006)
Hungarian meadow viper *(Vipera ursinii rakosiensis)*	Low Class 2	Genetic Bottleneck	?	(Ujvari et al 2002)
Fin Whale *(Balaenoptera physalus)*	Low Class 2	Exposure to Few Pathogens	moderate	(Trowsdale et al 1989)
Sei Whale *(Balaenoptera borealis)*	Low Class 2	Exposure to Few Pathogens	moderate	(Trowsdale et al 1989)
Malagasy jumping rat *(Hypogeomys antimena)*	Low Class 2	Bottleneck, mating system	low	(Sommer et al 2002a) (Sommer 2003)
Australian Bush rats *(Rattus fuscipes greyii)*	Low Class 2	Genetic Bottleneck,	?	(Seddon & Baverstock, 1999
Common Hamster *(Cricetus cricetus)*	Low Class 2	Genetic Bottleneck	?	(Smulders et al 2003)

Table 1.1 continued Species with Low or No Polymorphism at MHC loci and Proposed Cause.

Name	MHC low variance	Proposed Cause	Variation at neutral loci	Reference
European Beaver (*Castor fiber*)	Monomorphic Class 1 & 2	Genetic Bottleneck	low	(Ellegren et al 1993)
Cheetah (*Acinonyx jubatus*)	Low Class 1	Genetic Bottleneck	low	(Yuhki and O'Brien 1990)
Asiatic Lion -Gir Forest (*Panthera leo persica*)	Monomorphic Class 1	Genetic Bottleneck	low	(Yuhki and O'Brien 1990)
Lion-Ngorongoro Crater (*Panthera leo*)	Low Class 1	Genetic Bottleneck	low	(Yuhki and O'Brien 1990)
Lion-Ngorongoro Crater (*Panthera leo*)	Low Class 1	Genetic Bottleneck	low	(Yuhki and O'Brien 1990)
Musk Ox - Sweden (*Ovibos moschatus*)	Monomorphic Class 2	Genetic Bottleneck	low	(Mikko et al 1999)
Fallow deer-Sweden (*Dama dama*)	Monomorphic Class 2	Genetic Bottleneck	low	(Mikko et al 1999)
Prezwalski Horse (*Equus ferus przewalskii*)	Low Class 2	Genetic Bottleneck	?	(Hedrick et al 1999)
Arabian Oryx (*Oryx leucoryx*)	Low Class 2	Genetic Bottleneck	?	(Hedrick et al 2000)
Bontebok (*Damaliscus pygargus*)	Low Class 2	Genetic Bottleneck	?	(van der Walt et al 2001)
Moose - Sweden (*Alces alces*)	Low Class 2	Ancient Bottleneck, Solitary lifestyle	moderate	(Ellegren et al 1996)
Big Horn desert sheep (*Ovis canadensis mexicana*)	Low Class 2	Genetic Bottleneck	low	(Hedrick et al 2001a)
Mountain Goats (*Oreamnos americanus*)	Low Class2	Ancient bottleneck	low	(Mainguy et al 2006)

Table 1.1 continued Species with Low or No Polymorphism at MHC loci and Proposed Cause.				
Name	**MHC low variance**	**Proposed Cause**	**Variation at neutral loci**	**Reference**
San Nicolas Island fox (*Urocyon littoralis dickeyi*)	Low Class 2	Genetic Bottleneck	?	(Aguilar et al 2004)
Spanish ibex, (*Capra pyrenaica*)	Low Class 2	Loss habitat, hunting Epidemic sweep	?	(Amills et al 2004)
Common Chimpanzee (*Pan troglodytes*)	Low Class 1	Epidemic sweep	?	(de Groot et al 2002)
Malagasy grey mouse lemur (*Microcebus murinus*)	Low Class 2	Genetic Bottleneck	?	(Schad et al 2005)
Giant Panda (*Ailuropoda melanoleuca*)	Low Class 2	Genetic Bottleneck	Varied results	(Wan et al 2006)

The Relationship of MHC and Disease.

It is now well appreciated that MHC plays a central role in resistance to infectious and autoimmune disease in vertebrates (Briles et al 1983; Hill et al 1994; Kaufman and Wallny 1996). There are many studies showing a relationship between MHC haplotypes and disease due to bacteria, virus and parasitic worms, in natural populations and laboratory conditions. See Table 1.2 for examples of species with known relationships between diseases and their MHC.

The MHC – disease relationship is influenced by many other factors (multifactorial) e.g., mice infected with malaria show the outcome is significantly influenced by MHC haplotype as well as the parasite clone and host gender (Wedekind et al 2005). Even in the highly studied human MHC (HLA) direct correlation between an infectious disease and an MHC gene is difficult to establish due to the multifactorial nature of many diseases and linkage disequilibrium often prevents an unambiguous identification of the disease-causing or disease-associated loci (Shiina et al 2004a). In the HLA relationships between some autoimmune diseases and particular haplotypes has been identified more

often than with infectious diseases. A successful use of MHC haplotypes to control an infectious disease was shown in a Zebu cattle herd against infection with Dermatophilosis (Maillard et al 2002). However even though prevalence of the disease was reduced from 0.76 to 0.02, Maillard et al. concluded that the condition was probably multigenetic and multifactorial.

Table 1.2 Examples of Relationships between Species, Disease and MHC.

Species	Disease	MHC relationship	Reference
Three-spined stickleback (*Gasterosteus aculeatus*)	14 Macroparasites	Heterozygote advantage	(Wegner et al 2003)
Chinook salmon (*Oncorhynchus tshawytscha*)	INHV (viral) infectious haematopoietic necrosis virus	Heterozygote advantage	(Arkush et al 2002)
Atlantic salmon (*Salmo salar*)	infectious salmon anaemia virus (ISAV)	Negative frequency dependent selection	(Grimholt et al 2003)
Gila topminnow (*Poeciliopis occidentalis*)	Fluke (Gyrodactylus turnbulli)	Heterozygote advantage	(Hedrick et al 2001b)
Soay sheep (*Ovis aries*)	Strongyle nematode	Negative frequency dependent selection	(Coltman et al 1999)
Gray mouse lemur (*Microcebus murinus*)	Nematode spp.	Negative frequency dependent selection	(Rudel 2004)
Yellow-necked mouse (*Apodemus flavicollis*)	8 Nematode spp.	Negative frequency dependent selection	(Meyer-Lucht and Sommer 2005)
Hairy-footed gerbil (*Gerbillurus paeba*)	2 Cestodes spp., 6 Nematodes	Negative frequency dependent selection	(Harf and Sommer 2005)
Striped mouse (*Rhabdomys pumilio*)	8 Nematode spp.	Heterozygote advantage	(Froeschke and Sommer 2005)
Congenic MHC mice	*Salmonella enterica, Listeria monocytogenes,*	Heterozygote advantage	(Penn et al 2002)
Humans (*Homo sapiens*)	Hepatitis B	Heterozygote advantage	(Thursz et al 1997)
Humans (*Homo sapiens*)	HIV	Heterozygote advantage	(Carrington et al 1999)
Humans (*Homo sapiens*)	TB (*Mycobacterium tuberculosis*)	Negative frequency dependent selection	(Vijaya Lakshmi et al 2006)
House sparrow (*Passer domesticus*).	Avian malaria	Negative frequency dependent selection	(Bonneaud et al 2006)
Chicken (*Gallus gallus*)	Rous sarcoma virus	Heterozygote advantage	(Schierman et al 1977)
Chicken (*Gallus gallus*)	Mareks disease virus	Negative frequency dependent selection	(Briles et al 1977)

In birds the linkage of MHC to infectious disease resistance or susceptibility appears to be stronger than in mammals, this was first seen with Mareks disease (virus) in chickens where haplotype B21 gave an individual 95% resistance and B19 100% mortality (Cole 1968; Briles et al 1977; Hess and Edwards 2002). The smaller avian MHC size may give rise to the stronger disease linkage and it does imply a more pronounced importance in avian conservation for MHC (Hess and Edwards 2002). But even in birds the complications presented by the multifactorial aspect of "disease" can be seen with a study that found no definitive link between MHC haplotypes of the wild house finch (*Carpodacus mexicanus*), and a recent bacterial epidemic due to *Mycoplasma gallisepticum*. This may be due to the bacteria causing decreased expression of the Class II B loci, which could have circumvented this aspect of the immune response (Hess et al 2007).

The MHC genes are an important part of the defence against parasites and it also affects and is affected by other diverse biological systems resulting in a complex system where identifying direct and simple relationships is difficult (Zelano and Edwards 2002). The total influences of an individual's MHC genes on phenotype are diverse, and range from odour profile up to an individual's overall condition, which in turn can affect the physiological resources available for such activities as display, territory acquisition and defence (Zelano and Edwards 2002). However the effects potentially have even more layers with interactions affecting interactions such as the amount of expression of hormones like testosterone can influence the adaptive immune system (Hillgarth and Wingfield 1997), and MHC haplotypes can influence the testosterone levels (Gerencer et al 1982; Larsen et al 2000).

Within an individual MHC polymorphism enables it to respond to a wider range of pathogens, than a monomorphic individual. Simkova et al. (Šimková et al 2006) showed in cyprinid fish a positive relationship between nucleotide diversity of the Class II B exon 2 and parasite species richness. They found that populations exposed to high parasite pressure, in terms of high parasite species richness, maintained a high genetic diversity allowing them to decrease their natural mortality rate. This supports the assertion that at a population level, MHC polymorphism enables a greater flexibility in response to diseases, and gives an increased chance of survival for the population. The

ability of a vertebrate to mount an effective response to antigens is due to it's MHC haplotype (Levine and Benacerraf 1965; Benacerraf 1981), in combination with other genetic and non-genetic factors. It must also be remembered the mounting of an immune response, a major physiological mechanism in host survival, does have a cost to the animal which it must meet (reviewed (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000)). Factors such as current health status of the animal and previous life history also affect the speed and effectivity of the immune response.

The potential importance of MHC polymorphism to the conservation of endangered species is discussed in Chapter 5.

Chapter 2

The Kiwi: Conservation & Disease.

The one process now going on that will take millions of years to correct is the loss of genetic and species diversity by the destruction of natural habitats. This is the folly our descendants are least likely to forgive us. Edward O. Wilson, Pg 121, 1984 .

Introduction

The Kiwi (*Apteryx spp.*) is a unique bird, endemic to New Zealand, but despite being held in high regard by its people all five kiwi species are threatened/endangered and require active management to sustain current population's levels. Much of the decline of the Kiwi (*Apteryx spp.*) is due to the arrival of people bringing new mammalian predators and causing a loss of habitat for these once numerous birds. The decline and fragmentation of the Kiwi (*Apteryx spp.*) populations generally, means an increased importance for informed management to prevent of further loss of genetic diversity and to understand their susceptibility to disease. Knowledge of disease epidemiology in species in the wild is generally poor, and information regarding the Kiwi's genetic potential and immunocompetence to deal with existing and emerging diseases is important for its preservation.

The Kiwi

New Zealand's Unique Avifauna

The evolution of New Zealand's unique fauna and flora began before the fall of the dinosaur with the separation of New Zealand from ancient Gondwana about 82 – 85 MYA (Cooper and Millener 1993; Gibbs 2006). Some of the extant endemic species like the Tuatara (*Sphenodon punctatus* and *Sphenodon guntheri*)((Daugherty et al 1990) and the Leiopelma frogs (*Leiopelma archeyi* and *Leiopelma hochstetteri*) (Bell 1994) are ancient refugees from this departure (Daugherty et al 1993). Other endemic species are believed to be the results of repeated long distance colonisation events after geographical separation, such as the New Zealand (NZ) Short-Tailed Bat *(Mystacina*

tuberculata) (Kirsch et al 1998) and the New Zealand Long tailed Bat (*Chalinolobus tuberculatus*) (Lin et al 2001).

Since its separation from Gondwana, the New Zealand archipelago has gone through periods of rising and falling sea levels (Gibbs 2006). This resulted in episodes of immersion with possibly only isolated island refugia remaining and subsequent periods when land bridges connected the now separated three main islands – North Island, South Island & Stewart Island (Cooper and Cooper 1995; Gibbs 2006). It has been suggested there could have been a total immersion of the New Zealand landmass during the Oligocene which supports more importance for long distance dispersal producing New Zealand biota (Pole 1994; Waters and Craw 2006).

It is thought many of New Zealand's endemic animals fill ecological niches occupied by mammals elsewhere in the world, exhibiting ecological niche shifts, gigantism, and extended life histories with low reproductive rates. All of these factors lead to vulnerability to human disturbance (Daugherty et al 1993). Prior to colonisation by humans, New Zealand had approximately 245 bird species breeding here (Holdaway et al 2001). Today, an estimated 1/3 are extinct, and half of the land based birds have restricted distribution and are threatened or endangered (Holdaway 1989; Wilson 2004).

Taxonomy & Distribution of the Kiwi

The Kiwi (*Apteryx spp.*) is the smallest member of the ratites (Order Struthioniformes). Ratites along with the Tinamous of South America constitute the Superorder Paleognathae. The Paleognathae are based on an archaic palatal morphology (bones forming the roof of mouth) and are traditionally considered the basal branch in modern birds. All other modern birds belong to the Neognathes. Some mitochondrial DNA analysis suggested the passerine/neornithes split was more basal than the paleognathae/neognathae divergence (Mindell et al 1997; Härlid 1998; Harlid and Arnason 1999; Mindell 1999). However more recent avian mitochondrial analysis supports the paleognathae/neognathae split (92-101 MYA) as basal (Harrison et al 2004).

The exact relationship amongst ratite species is not well established (Worthy and Holdaway 2002). Analysis of mitochondrial DNA has shown that the relationship

between the two New Zealand ratites, the extant kiwi and the extinct moa, was not as close as previously thought (Cooper et al 1992). In these results the kiwi showed a closer relationship to the Australian Emu (*Dromaius novaehollandiae*) and Cassowary (genus *Casuarius)*), and even the African Ostrich (*Struthio camelus*) than to the moa (Cooper et al 1992). See Fig E.2. The separation of the kiwi from the Emu and Cassowary, unlike the moa, has been calculated to be after the separation of New Zealand from Gondwana (Cooper et al 1992; Cooper et al 2001). This leaves unanswered when and how did the kiwi travel across the divide to New Zealand.

The kiwis are the only known members of the genus *Apteryx* and the family Apterygidae. Starting in 1813, there have been various taxonomical divisions of the extant genus *Apteryx*. Until the genetic studies of the 1990s the kiwi taxonomy was mainly based on work by Mathews (Mathews 1931; Mathews 1935) and Oliver (1930) and defined as much by geography as morphology with brown kiwi split into North Island, South Island and Stewart Island. Currently there is support for *Apteryx* to be divided into five species, but up to ten different species have been suggested in the past (Herbert and Daugherty 2002). It is difficult to differentiate between kiwi species morphologically due to a lack of distinguishing features when dealing with fossils and bones. Baker et al. (1995) suggested the difficulties arose from using morphological features that were phylogenetically primitive and uninformative for differentiating *Apteryx spp*. Without molecular biological testing, only the bones/fossils of the Little Spotted Kiwi, which has a smaller general body size can be confidently distinguished from other kiwi, i.e., the Greater Spotted and the Brown Kiwis (Worthy 1998a).

The extant *Apteryx* genus is presently recognised as containing five species (Burbidge et al 2003; Committee 2004). The basal division is between the Spotted Kiwis and the Brown Kiwis. The Spotted Kiwis consists of the Great Spotted Kiwi (*Apteryx haastii*) and the smaller Little Spotted Kiwi (*Apteryx owenii*). The Brown Kiwis are an allopatric group, divided into the North Island Brown (*Apteryx mantelli*), the Tokoeka (*Apteryx australis*), and the more recently identified Rowi (*Apteryx rowi*) (Burbidge et al 2003; Tennyson et al 2003). It has been suggested that a small isolated population of Tokoeka in the Haast area may represent a 4th Brown Kiwi species (Herbert and Daugherty 2002). An extinct variant Brown kiwi from Eastern South Island has also been proposed

as a separate species (Worthy 1997, 1998a, 1998b). The current distribution of the each *Apteryx* spp. is shown in Figure 2.1 and Table 2.1.

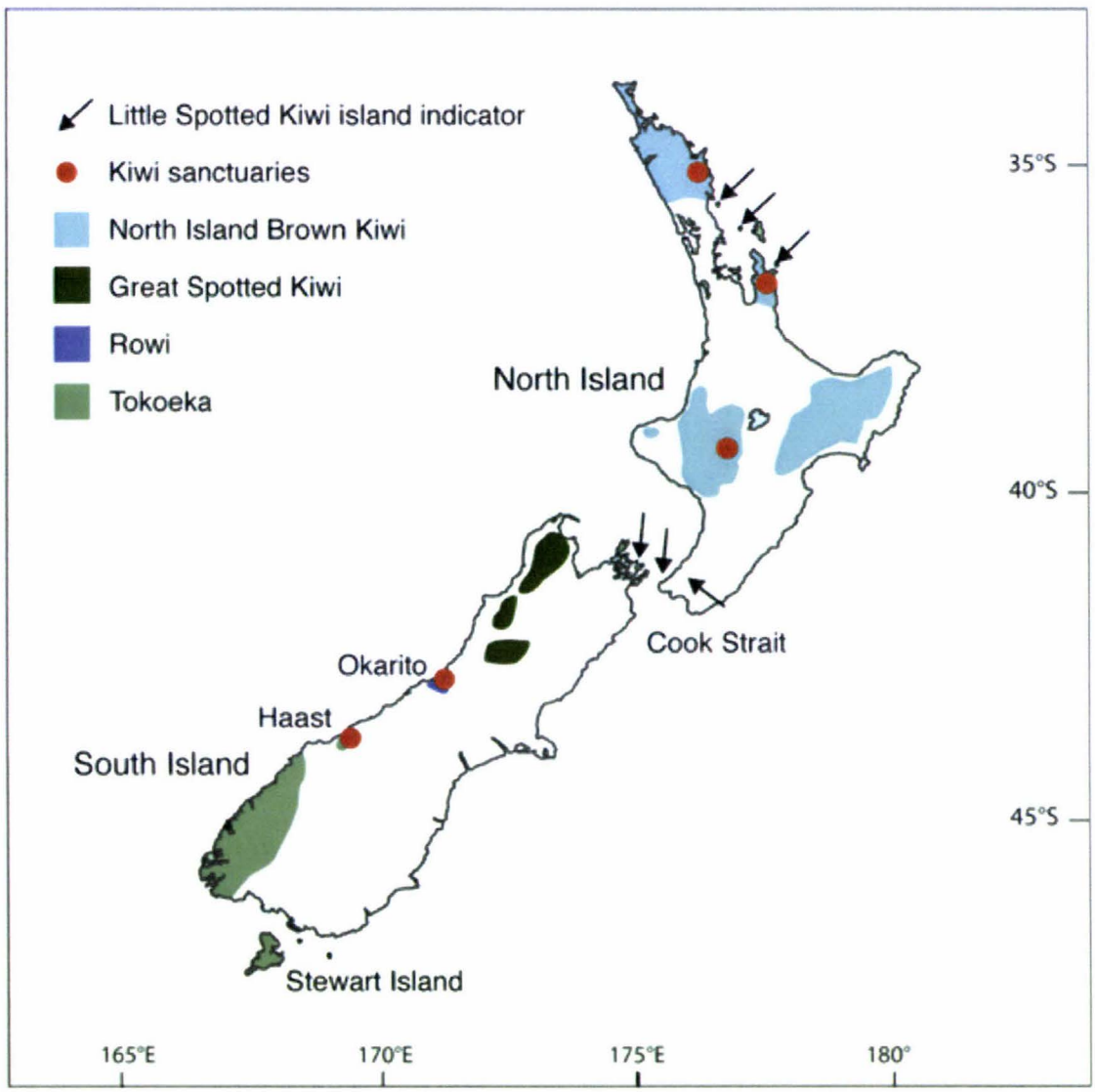


Figure 2.1 Current distribution of the five kiwi species, and the five DOC kiwi sanctuaries for more intensive management of kiwis. n.b. in this diagram the kiwi sanctuary at Okarito encompasses the known rowi distribution. Reproduced with the kind permission of L.D. Shepherd (Shepherd 2006) and redrawn by Vivian Ward..

Past distribution of each *Apteryx* spp. is difficult to establish due to the similarity of bones and the gaps in the fossil/subfossil records (Baker et al 1995). The Little Spotted Kiwi once was numerous on both the North and South Islands but now only found on five off-shore islands and in one mainland sanctuary. The Great Spotted Kiwi is in three fragmented populations and is showing a slow declining in population size. The once

numerous North Island Brown covered the top 2/3 of the North Is. and is now generally retreating in distribution and numbers. The Rowi is currently only found in a single small isolated population at Okarito in the South Island. Analysis of ancient DNA in bones has raised the suggestion of rowi previously existing in the north-west of the South Is. and the south-east of the North Is (Shepherd 2006). The Tokoeka is gradually declining even though most of the habitat in Fiordland and Stewart Island is protected. Previously Tokoeka also extended over Southland, Otago and Canterbury i.e. east of the Southern Alps. The current Department of Conservation (DOC) and IUCN conservation status of the Kiwi and their distribution is listed in Table 2.1. The general picture shows an ongoing need for active management based on sound research to reverse the decline and possible extinction of the *Apteryx* species.

The Retreat of the Kiwi. – Past and Present.

"The birds of New Zealand are noted for two things – their uniqueness and their rapid disappearance" (Meyers 1923)

The arrival of people to New Zealand, and their entourage, has been called "an ecological holocaust" (Clout and Craig 1998). Prior to the arrival of humans about 78% of the land area of the main islands of New Zealand from coast to tree line, was covered with forest which is now reduced to a fragmented 23% (Clout and Craig 1998). Despite more than 100 years of legal protection of kiwi (1896–present day), all kiwi are under threat, e.g., unmanaged North Island Brown Kiwi populations are declining at 5.8% per annum (McLennan et al 1996). Most of the decline in kiwi populations has been attributed to introduced predators, especially attacking the vulnerable juveniles during the first six months of life (McLennan and Potter 1993). The kiwi predators introduced by man to New Zealand are: Cat (*Felis catus*), Dog (*Canis familiaris*), Ferret (*Mustela furo*), Pig (*Sus scrofa*), Possum (*Trichosurus vulpecula*), Stoat (*Mustela erminea*) and the Weasel (*Mustela nivalis vulgaris*). McLennan et al. (1996; 2004) suggested that ferrets and dogs are the main predators of adult kiwi, possums and mustelids are the main egg predators, while stoats and cats are largely responsible for the deaths of young kiwi. These researchers estimated the current total juvenile mortality rate of about 94% in unmanaged populations, could be reduced by about 1/3 with the removal of these predators. However, a study of Little Spotted Kiwi (LSK) on Kapiti Island which has

no introduced predators, estimated 60% of loss of the young were due to predation by the endemic weka (*Gallirallus australis*) (Jolly 1989). Which suggests not all predation is introduced.

Loss of habitat adversely impacts on the kiwi in a variety of ways. The clearing of native forest causes loss of life due to burning and roller crushing. The remaining kiwi, which are highly territorial (territory size range 2–100 Ha), are then concentrated in the remaining habitat. This results in presumably less resources per head and more territorial disputes, possibly less breeding as well as closer proximity to predators. As a flightless bird with limited dispersal ability (Burbidge et al 2003), fragmentation of populations from loss of habitat, increases their genetic isolation and may lead to inbreeding.

The extent of the loss of genetic diversity in brown kiwi is shown in work comparing cytochrome b haplotypes in modern kiwi and ancient bones. Seventeen of the 44 brown kiwi haplotypes (38.6%) were only detected in ancient populations (Shepherd 2006). The study showed losses of overall genetic variation in North Island brown kiwi of 14.2%, 66.6% loss in rowi and 42.8% loss in tokoeka (Shepherd 2006).

Table 2.1 Species of kiwi with their present distributions and conservation status. Courtesy of L.D. Shepherd (Shepherd 2006) with IUCN Red list Category included.

Species	Current Distribution[1]	Current population estimate[1]	Conservation status[2, 3.]
North Island brown kiwi (*Apteryx mantelli*) 		25 000	Seriously declining[2] Endangered.[3]
Rowi (*Apteryx rowi*) 		250	Nationally critical [2] Not classified.
Tokoeka (*Apteryx australis*) 		300 at Haast and 30-33 000 in Fiordland and Stewart Is.	Gradually declining[2] Vulnerable[3]
Little spotted kiwi (*Apteryx owenii*) 		1 200 on Kapiti Is. and a total of 200 on other islands and mainland sanctuaries.	Range restricted[2] Vulnerable[3]
Great spotted kiwi (*Apteryx haastii*) 		17 000	Gradually declining[2] Vulnerable[3]

[1]Bank of New Zealand Kiwi Recovery™ Trust. http://www.kiwirecovery.org.nz
[2]Hitchmough (2002) [3] The International Union for the Conservation of Nature and Natural Resources. (IUCN) Red List Category. http://www.iucnredlist.org/

Introduced diseases have been suggested as contributing to the reduced state of Kiwi and NZ avifauna generally, but there is little substantiating evidence available (Meyers 1923; Moon 1988; Gill 1999). The rapid decline of once numerous species, even in

areas considered to be untouched by humans and introduced predators, raised the index of suspicion e.g., the decline of bellbird in the North Island in 1862 (Williams 1973), but no "smoking gun" evidence has been found. Some work by Dore suggested the decline of the kiore (*Rattus exulans*) could be due to the introduction of the blood parasite *Trypanosoma lewisi* from European rats (Dore 1918). Introduced strains of avian malaria could have affected some NZ birds (Dore 1920), it is certainly implicated in the decline of the Hawaiian land birds (van Riper et al 1986; Jarvi et al 2004). In a review, De Castro & Bolker (2005) could not find direct or unequivocal empirical evidence for the complete extinction of a species in the wild due to a pathogen. However they did conclude that careful consideration of all possible factors is important, especially acting in combinations e.g. situations with a small pre-epidemic population size and a reservoir host for the pathogen were often implicated in putative disease related extinctions.

Kiwi Conservation

The Kiwi is of cultural as well as biological importance, it is a taonga (treasure) to Mäori and to modern New Zealand society. Significant co-ordinated conservation management action started in 1991 with a Kiwi Recovery Programme (Butler et al 1991; Robertson 2003). The ten year plan by the Department of Conservation (1996-2006) had a long term goal of kiwi recovery, to maintain and where possible, enhance the current abundance, distribution and genetic diversity of the kiwi (DOC 2003). Various research and predator control programmes were initiated for this purpose with emphasis on predator control, which is recognised as the main cause of kiwi decline (McLennan et al 1996; Basse et al 1999; Pierce and Westbrooke 2003). To maintain current mainland populations it is estimated that juvenile survival rates need to be raised in some areas from 6% to 19% (McLennan et al 1996). Management initiatives like Operation Nest Egg (ONE) involve incubating eggs and raising juveniles kiwis in captivity until they are able to fend off most predators (800-1200g body weight) and then they can be returned to the wild (Grant 2001; Committee 2004; McLennan et al 2004). Five kiwi sanctuaries have also been established, to protect populations of particular importance (Committee 2004). Figure 2.1 shows locations of sanctuaries and current Kiwi distribution.

Current planning by the New Zealand Department of Conservation (DOC 2007), recognises 11 varieties of kiwi, each requiring separate management plans: North Island Brown kiwi (NIB) (4 varieties), Little Spotted Kiwi (LSK), Great Spotted Kiwi (GSK) (Roroa), Okarito brown kiwi (Rowi), Haast Tokoeka, and Southern Tokoeka (three varieties- Stewart Island, Southern Fiordland, Northern Fiordland). The draft goals include more research into genetics, taxonomy and diseases of kiwi, but the main emphasis is predator control (DOC 2007).

Genetic factors such as loss of variability and heterozygosity can impact on a species' road to extinction (DeSalle 2005). Several kiwi populations (Rowi, Haast Tokoeka, LSK on small islands e.g., Red Mercury) are very small and isolated which could lead to inbreeding and loss of genetic diversity unless actively managed. Inbreeding depression can be a factor in extinction, especially in island populations like New Zealand land birds (Frankham 1998). Although it is difficult to predict how a given population will respond to inbreeding especially under variable environmental conditions (Hedrick and Kalinowski 2000), depression of immune response can result from inbreeding (Reid et al 2003; Hale and Briskie 2007). Generally inbreeding depression increases under stressful conditions (Armbruster and Reed 2005) making interventions at this late stage more difficult. Spielman et al. (2004) showed with a meta-analysis approach that inbreeding and loss of genetic diversity decreased disease resistance. This was due to specific polymorphic loci affecting disease resistance, rather than a consequence of generalised inbreeding depression (Spielman et al 2004). The importance of diversity at, the Major Histocompatibility Complex (MHC) to conservation and disease resistance is discussed in chapters 1 & 5. Inbreeding depression effects are expected to be greater for natural populations as they are exposed to many different pathogens. Inbred populations that are relatively resistant to one pathogen are likely to be susceptible to most other unrelated pathogens (Spielman et al 2004).

The Importance of Infectious Disease in Conservation.

Recognition of the role of disease as a limiting factor in wildlife survival is increasing (Deem et al 2001). Unfortunately we generally have only limited knowledge about diseases, pre-existing or emerging in wildlife, making analysis of the risks they pose difficult (Jackson et al 2000; Jakob-Hoff 2000). The effects may be obvious with

catastrophic loss of species in a community, e.g., an outbreak of avian cholera (*Pasteurella multocida)* infection in Saskatchewan killed >4,900 ducks, particularly redheads *(Aythya americana)* concentrated for moulting (Wobeser 1992; Snyder et al 1996). However the effects may also be more subtle such as when a new pathogen that causes a persistent sub-lethal infection is hosted and spread by an invading species. This may be an important factor in an invading species overcoming an established but naive endemic competitor or in the reverse situation may keep out the naïve invading species (Prenter et al 2004). Such sub-lethal infections would not only be difficult to identify, but their contribution to an animals and its offspring's fitness may depend on other compounding factors. There is concern about our ability to diagnose and control emerging diseases in wildlife, as failure to do so will result in our inability to sustain current abundance and distribution of some wild bird species including threatened avifauna (Friend et al 2001).

Assuming we do have the ability to diagnose and treat a disease in wildlife, it may not be enough to enable sustainable management of a wild population (McCallum and Dobson 1995), as illustrated in the growing problem of worm control in domestic sheep. Sheep management in New Zealand and overseas has relied on pharmaceutical control of gastrointestinal parasites with Anthelmintics (drenches). More than 50% of sheep farms now have detectable levels of resistance to one or more chemical classes of anthelmintic (Leathwick et al 2001). Our current treatment paradigm is found wanting and we have created the current problem from our reliance on anthelmintic use. A new approach in domestic sheep called "integrated parasite or pest management" is now proposed for sustainable management based on a wider perspective of host- parasite interactions (Besier 2002). The host-parasite relationship exists as a system embedded within larger systems represented by ecological communities and ecosystems and these complex systems are each dynamic and co-evolving (Horwitz and Wilcox 2005). Based on this paradigm Horwitz and Wilcox (2005) proposed six principals for integrated pest or parasite management :

1. Ensuring the strategic application of chemical controls and when used is in combination with other practices.
2. Management of any reservoir populations (e.g., other host species of parasite) to minimise reinfection.
3. Management of habitat to maximise the effect of other biota on the parasite.

4. Help the ability of the host to resist infection through its well being, nutritional status and/or immune system i.e., minimise stress and maximise immunocompetence.
5. Close monitoring of all cross-scale components of a system to know when and where to implement or modify the approach, i.e. get feedback from the system to evaluate response to interventions.
6. Critical attention must be given to human based disturbances that result in ecosystem level changes and may result in emergent properties like new parasites/pathogens.

The production of an integrated management plan for a wild population, like the kiwi, will therefore require in-depth knowledge of the birds, their parasites/pathogens and interactions with the surrounding ecosystem. It is likely the management programme for each population will differ, however the need for pertinent immunocompetence and immunogenetics data on each population, including but not only MHC polymorphism, exists (Acevedo-Whitehouse and Cunningham 2006).

The Importance of Disease to Apteryx spp.

Like most wildlife, there is only a limited amount of information known about the diseases of kiwi (Jakob-Hoff 2000). Most of this information is from captive birds, with little work available on the disease epidemiology in the wild. Table 2.2 shows the parasites and pathogens recorded in Kiwi and Table 2.3 shows some of the diseases associated with infection found in the Kiwi. The effect of a given parasite on its kiwi hosts' ability to survive and reproduce i.e., it's fitness has yet to be established. Unfortunately, even if we had a much fuller knowledge of present kiwi pathogens and their pathogenicity, the emergence of new diseases i.e., new challenges cannot be excluded.

Emerging infectious diseases (EID), have been gaining recent prominence e.g. West Nile Virus, Severe Acute Respiratory Syndrome (SARS) and Avian influenza (AI). They result from complex interactions with social, physical, chemical, and biological dimensions of our planet's systems (Wilcox and Colwell 2005) making estimating their potential effect on a threatened species like the kiwi problematic. Due to New Zealand's isolation, the two main methods for arrival of such diseases/pathogens from overseas are human importation (deliberate and accidental) and biological translocation such as migrating birds. To stop the importation of harmful animal diseases but at the same time

to allow for international trade, the OIE (Office international des épizooties) sets standards. These allow for the importation risk analysis of diseases by national authorities but limit the reviews to already identified diseases. The flaw in this approach was shown by the mass marine mortalities of Australian pilchards (*Sardinops sagax*) due to a previously unknown herpes virus from imported fish food (Gaughan 2001). Relying on diseases already being identified in a world of evolving pathogens and previously unexposed species is an ongoing risk. Work was done prior to the (illegal) introduction of rabbit calci virus to NZ on its effect on the Kiwi (Buddle et al 1997). However this is the exception, typically there is not a great deal of information to make informed decisions about the affect of an EID on a threatened species. We need a much greater understanding of the kiwi and its pathogens in general and its immunocompetence (including MHC polymorphism) in particular to understand the effects of known diseases and potential new ones. A population which is genetically depauperate at the MHC level is likely to be more at risk to infectious disease (O'Brien and Evermann 1988).

We can resort (in extremis) to assuming that if a pathogen infects a closely related species then there is a risk of infection transmission and disease to the Kiwi. The Kiwi does not have close living relative at or below Family level. However, likely available indicators of potential pathogens would be other ratites (Order Struthioniformes) such as the Ostrich and the Emu. Using the assumption that relatedness implies disease susceptibility the Kiwi may also be susceptible to avian influenza. The recent (and ongoing) avian influenza pandemic has caused concern as farmed ostrich in South Africa have previously been affected by the AI virus (Huchzermeyer 2002). See Table 2.4 for known diseases of Ratites. Farmed ratites like the Ostrich and Emu have more information available on diseases than the undomesticated Kiwi (*Apteryx spp.*).

Table 2.2. Parasites and Pathogens found and recorded in Kiwis.

Parasite Species	**Kiwi spp.**	**Reference**
Feather lice		
Apterygon mirum	NIB	(Clay 1961; Tandan 1972)
Apterygon dumosum	Tokoeka, LSK	(Tandan 1972)
Apterygon hintoni	GSK	(Clay 1966; Tandan 1972)
Apterygon okarito	Rowi	(Palma and Price 2004)
Rallicola gadowi	Tokoeka	(Clay 1972)
Rallicola gracilentus	GSK	(Clay 1972)
Rallicola pilgrimi	LSK	(Clay 1972)
Rallicola rodericki	NIB	(Palma 1991)
Rallicolla sensulato		(Orr 1997)
Feather mites		
Kiwialges palametrichus	NIB, LSK, GSK	(Gaud and Laurence 1981; Bishop 1984)
	Tokoeka,	(Gaud and Atyeo 1970; Bishop 1984)
Kiwialges phalagotrichus	NIB, GSK	(Bishop 1984)
	Tokoeka, LSK	(Gaud and Atyeo 1970; Bishop 1984)
Kiwialges cryptosikyus	Tokoeka	(Gaud and Atyeo 1970; Bishop 1984)
	GSK	(Bishop 1984)
Kiwialges haastii	GSK	(Bishop 1984)
Ticks		
Haemaphysalis longicornis	NIB	(Heath 1977)
Ixodes anatis	NIB	(Dumbleton 1953)
Fleas		
Parapsyllus nestoris nestoris	Tokoeka	(Smit 1979)
Haematozoa		
Babesia kiwiensis	NIB	(Jakob-Hoff et al 2000; Pierce and Westbrooke 2003)
Hepatozoon kiwii	NIB	(Jakob-Hoff et al 2000; Pierce and Westbrooke 2003)
Plasmodium spp.	NIB	(Boardman 2000)
Trematodes		
Lyperosomum megacotylosum	NIB	(Andrews 1977)
Cestodes		
Cestode spp.	NIB	(Alley and Gartrell 2003)
Nematodes		
Cyrnea apterycis	NIB	(Harris 1975; Clark and McKenzie 1982)
Heterakis apterycis	NIB	(Clark and McKenzie 1982)
Heterakis gracilicauda		(Boardman 1998a)
Toxocara cati	NIB	(Clark and McKenzie 1982)
Porrocaecum ensicaudatum	NIB	(Clark. 1983)
Cupillaria sp.	NIB	(Clark. 1983)
Tetrameres sp.	NIB	(Clark. 1983)
Syngamus sp		(Jakob-Hoff 1998)

Table 2.2. continued Parasites and Pathogens found in kiwis.

Pathogen Species	Kiwi spp.	Reference.
Protozoa		
Eimeria spp. (2?)	NIB	(Thompson and Wright 1978; Boardman 1994)
Toxoplasma sp(suspected Atoxoplasma sp)		(Boardman 1998b)
Fungi		
Cryptococcus neoformans var. gattii	NIB	(Hill et al 1995; Alley 2001)
Aspergillus sp	NIB	(Boardman 1994)
Bacteria		
Pasturella multocida		(Reece and Hartley 1994)
Salmonella typhimurium		(Boardman 1994)
Proteus mirabilis		(Boardman 1994)
E.coli		(Boardman 1994)
Erysipelothrix rhusiopathiae	LSK	(Black and Orr 1996)
Mycobacterium avium	NIB	(Davis et al 1984; Boardman 1998b) .

Table 2.3 Diseases associated with infections* recorded in the Kiwi.(*Apteryx spp.*).

Disease	**Aetiology**	**Frequency**	**Reference**
Egg yolk peritonitis	Due to *E. coli* infection	Common	(Haigh 1994)
Yolk sac retention	Due to *E. coli*, Proteus, or, Streptococcus infection.	Common	(Boardman 1998b)
Septicaemia	Due to *Salmonella typhimurium, Proteus mirabilis, E. coli, Pasteurella multocida,* infection	Common	(Boardman 1998b)
Aspergillosis - Mycotic pneumonia and air sacculitis	Aspergillus sp	Common	(Boardman 1998b)
Coccidiosis	Eimeria spp.(more than one suspected)	Common	(Thompson and Wright 1978)
Trauma	Aggression between kiwi can result in wounds and infection.	Common	(Boardman 1998b)
Nematodiasis	Cyrnea aptericis, Heterakis gracilicauda.	Common	(Boardman 1998b)
Vestibular Disease	Unknown but antibiotic responsive implies infection	Occasional	(Jakob-Hoff 1997)
Pneumonia, bronchitis	Pasteurella multocida, Aspergillus sp., E. coli, virus? Others	Occasional	(Clemance 1997; Boardman 1998b)
Babesiosis	Babesia sp	Common	(Jakob-Hoff et al 2000)

Table 2.3 continued Diseases associated with infections* recorded in the Kiwi.(*Apteryx spp.*).			
Disease	**Aetiology**	**Frequency**	**Reference**
Proventriculitis	?	Common?	(Huia 2003)
Air sacculitis	?	Occasional	(Huia 2003)
Necrotic enteritis	Unknown	Occasional	(Boardman 1998b)
Hepatitis	?	Occasional	(Lintott 1994),
Renal Necrosis	?	Uncommo n	(Huia 2003)
Cryptococcosis	*Cryptococcus neoformans,* associated with Eucalyptus leaf substrate	Rare	(Hill et al 1995)
Cerebral nematodiasis	Unidentified nematode	Rare	(Boardman 1998b)
Toxoplasmosis (suspected)	*Toxoplasma gondii*	Rare	(Boardman 1998b)
Avian TB	*Mycobacterium avium*	Rare	(Davis et al 1984)
Hepatozoonosis	Hepatozoon sp	Rare	(Jakob-Hoff 2000)
Avian malaria	Plasmodium spp.	?	(Boardman 2000)
Chronic necrotizing colitis	?	?	(Huia 2003)
Ventriculitis	Parasites, other	?	(Huia 2003)
Parasitic hepatitis	?	?	(Huia 2003)
Acute colitis	?	?	(Huia 2003)
Cellulitis	Bacterial infection, trauma	?	(Huia 2003)
Myositis	?	?	(Huia 2003)

*probable infections.

Table 2.4 Recorded Infectious Diseases of Ratites.

Parasite/Pathogen Species	**Reference**
Feather lice	
Struthiolipeurus nandu	(Ponce Gordo et al 2002)
Struthiolipeurus rheae	(Ponce Gordo et al 2002)
Feather mites	
Dermoglyphus pachycnemis	(Ponce Gordo et al 2002)
Gabucinia bicaudata	(Ponce Gordo et al 2002)
Ticks	
Ixodid spp.	(Mertins and Schlater 1991)
Argasid spp.	(Mertins and Schlater 1991)
Helminths (Nematodes, Trematodes, Cestodes)	
Syngamous trachea	(Ponce Gordo et al 2002)
Libiostrongylus douglassii	(Barton and Seward 1993; Ponce Gordo et al 2002)
Libiostrongylus magnus	(Ponce Gordo et al 2002)
Libiostrongylus dentatus	(Hoberg, Lloyd, & Omar, 1995)
Trichostrongylus tennuis	(Ponce Gordo et al 2002)

Table 2.4 continued Recorded Infectious Diseases of Ratite.

Parasite/Pathogen Species	Reference
Helminths (Nematodes, Trematodes, Cestodes)	
Houttuynia struthionis	(Pintori et al 2000)
Philophthalmus gralli	(Ponce Gordo et al 2002)
Unknown trematode	(Ponce Gordo et al 2002)
Codiostomum struthionis	(Jansson and Christensson 2000; Ponce Gordo et al 2002)
Capillaria spp.	(Ponce Gordo et al 2002)
Capillaria parvumspinosa	(Ponce Gordo et al 2002)
Ascarid spp.	(Ponce Gordo et al 2002)
Helminths (Nematodes, Trematodes, Cestodes) continued	
Ascaridia struthionis	(Ponce Gordo et al 2002)
Baylisascaris procyonis	(Shane 1998)
Baylisascaris columnaris	(Shane 1998)
Chandlerella quiscali	(Shane 1998)
Fungi	
Aspergillosis spp.	(Shane 1998)
Basidia spp.	(Shane 1998)
Rhizopus spp.	(Shane 1998)
Mucor spp.	(Shane 1998)
Candidia spp.	(Shane 1998)
Trichophyton spp.	(Shane 1998)
Protozoa	
Cryptosporidium sp	(Gajadhar 1993)
Coccidia Isospora sp	(Jansson and Christensson 2000)
Coccidia Eimeria sp	(Sotiraki et al 2001)
Balantidium struthionis	(Sotiraki et al 2001)
Entamoeba spp.	(Jansson and Christensson 2000)
Endolimax sp.	(Ponce Gordo et al 2002)
Iodamoeba sp	(Ponce Gordo et al 2002)
Histomonas meleagridis	(Ponce Gordo et al 2002)
Monocercomonas sp.	(Ponce Gordo et al 2002)
Tetratrichomonas gallinarum	(Ponce Gordo et al 2002)
Trichomonas gallinae	(Ponce Gordo et al 2002)
Giardia sp.	(Ponce Gordo et al 2002)
Spironucleus meleagridis	(Ponce Gordo et al 2002)
Chilomastix gallinarum	(Ponce Gordo et al 2002)
Retortamonas sp.	(Ponce Gordo et al 2002)
Pleuromonas jaculans	(Ponce Gordo et al 2002)
Euglenid flagellate.	(Ponce Gordo et al 2002)
Blastocystis sp ?	(Stenzel et al 2004)
Leukocytozoon sp	(Shane 1998)
Plasmodium sp	(Shane 1998)
Aegyptianella sp	(Shane 1998)
Hexamita sp	(Shane 1998)

Table 2.4 continued Recorded Infectious Diseases of Ratite.

Parasite/Pathogen Species	Reference.
Bacteria	
Haemophilus spp.	(Shane 1998)
Salmonella spp.	(Shane 1998)
Salmonella pullorum	(Shane 1998)
Salmonella arizonae	(Shane 1998)
Bacillus anthracis	(Shane 1998)
Campylobacter jejuni	(Shane 1998)
Clostridial spp.	(Shane 1998)
Cl. Perffringens	(Shane 1998)
Spirochete spp.	(Shane 1998)
Mycoplasma spp.	(Huchzermeyer 2002)
Staphylococcus hyicus	(Shane 1998)
Bacteria	
Erysipelothrix rhusiopathiae	(Shane 1998)
Mycobacterium avium	(Shane 1998)
Chlamydia	(Shane 1998)
Pasteurella multocida	(Shane 1998)
Esherichia coli	(Shane 1998)
Virus	
suspected Adenoviruses	(Shane 1998)
suspected Circoviruses	(Shane 1998)
suspected Arenaviruses	(Shane 1998)
Coronaviruses	(Shane 1998)
Ostrich pox virus	(Shane 1998)
Avian influenza virus – orthomyxovirus	(Huchzermeyer 2002)
Newcastle disease virus	(Huchzermeyer 2002)
Western Equine Encephalitis- Alphavirus	(Shane 1998)
Bornavirus	(Shane 1998)
Eastern Equine Encephalitis- Alpha togavirus	(Shane 1998)
Adenovirus	(Shane 1998)
Crimean-Congo Haemorrhagic fever- Arbovirus	(Shane 1998)
Infectious Bursal Disease (IBD)- Avibirnavirus-	(Shane 1998)

Chapter 3

Materials & Methods.

3.0 Introduction.

This Chapter details the different methods and materials used at various stages of the project. They are listed in the following order:

3.1 Samples – source of kiwi DNA samples

3.2 DNA & RNA extraction from samples.

3.3 PCR – details of PCR's used to amplify MHC sequences together with the details and conditions for their sequencing.

3.4 Agar Gel Electrophoresis (AGE)

3.5 PCR purification techniques- a list of methods used to extract the DNA product from the resulting PCR reaction.

3.6 Cloning techniques – a list of the methods used to amplify plasmids with target DNA.

3.7 MiniPrep Techniques – a list of the methods used for extraction of plasmid DNA from bacteria.

3.8 Nanodrop – a technique using ND-1000 Spectrophotometer to estimate DNA concentrations.

3.9 Agarose Gel Extraction Techniques (AGE) - methods used to extract DNA from Agarose.

3.10 TempliPhi™ - a method for rolling amplification of plasmids.

3.11 ExoSAP- a technique used to digest ssDNA and unused dNTPs.

3.12 Sequencing – the techniques used to sequence DNA using the Big Dye Termination mix 3.1 (BDT 3.1) in an ABI 3730 Genetic Analyzer.

3.13 Agar Plates – a list of the different types of agar plates used.

3.1 Samples – sources of Kiwi DNA samples.

3.1.1 DNA from feathers. Feathers were used as the source of DNA, as it is non-invasive to collect, and can be a source of good quality DNA. The Kiwi feather samples

were supplied by the New Zealand Department of Conservation (DOC) from a population of North Island Brown kiwi (NIB) near Whangarei, and a population of Little Spotted Kiwi (LSK) on Red Mercury Island. The Okarito Brown Kiwi (Rowi) samples were supplied from the Rowi population in Okarito by Dr. Karen J. Nutt of Waikato University and DOC. See Figure 5.1. for the location of the 3 populations sampled. Once collected the feather samples were kept in labelled paper envelopes at room temperature.

3.1.2 RNA from Blood. A sample of Kiwi blood was collected from a North Island Brown (NIB) at Auckland Zoo, while it underwent a routine veterinary examination. Two drops of blood were immediately put into a plain blood collection tube containing Trizol (Invitrogen) and stored at 4°C. From this mRNA was extracted (see section 3.2.2) and used to synthesise cDNA which in turn was used in a 5′ RACE PCR (see section 3.3.3).

3.2 DNA & RNA extraction from samples.

3.2.1 DNA extraction from feathers - The bottom 3-4 mm of 2-3 feathers of a single bird, i.e., the follicle, was removed and placed in a 1.5 ml Eppendorf tube with 200 µl of SET buffer (100 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA). Add 10µl of Proteinase K (20 mg/ml in water), 5 µl of 1 M Dithiothreitol (DTT), and 10 µl of 20% SDS. The mixture was incubated on a rotator overnight at ~55°C. Equal volumes of Phenol: Chloroform: Isoamyl Alcohol are added and mixed. It was then centrifuged at RCF = 15,000g. The top 160 µl are put in a fresh tube with 80 µl 7.5M ammonium acetate, and 500 µl 100% ethanol, and then mixed. The mixture was next put on ice for 10 minutes, then centrifuged at RCF = 15,000g. After carefully decanting the liquid, 400 µl 70% ethanol was added and mixed. Centrifuge for 1minute at RCF = 15,000g. Remove as much ethanol as possible, and resuspend in ~30 µl of milliQ water and stored at 4°C overnight. Once extracted the DNA was stored in -80°C freezers if not used within 24 hours.

3.2.2 mRNA extraction from Kiwi blood.

The mRNA was extracted from the Kiwi blood using Invitrogen Trizol ® as per manufacturers' instructions. It was then dissolved in RNA-free dH_2O and stored at -80°C until used.

The Trizol technique is a 2 step modification of the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). Trizol is a monophasic solution of phenol and guanidine isothiocyanate. The blood cells are lysed, but the integrity of the RNA is maintained. Then the addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. The RNA is recovered by precipitation with isopropyl alcohol. The RNA can then be washed in 75% ethanol then gently dried until the ethanol has evaporated. At this point the RNA can be dissolved in RNA-free dH_2O and either stored or used. The DNA and proteins in the sample can be later recovered from the organic phase.

3.2.3 Phenol: Chloroform Nucleic Acid Extraction.

The Phenol Chloroform method for Nucleic Acid Extraction was used as described by Sambrook in Molecular Cloning: A Laboratory Manual (Sambrook et al 1989) using Phenol: Chloroform: Isoamyl Alcohol instead of Phenol: Chloroform.

3.3 Polymerase Chain Reaction (PCR).

To obtain the Kiwi MHC DNA sequences identified in this project a range of previously published primers (see Table 3.1) and primers designed specifically for this project (see Table 3.2) were used. A large range of modifications were also made to the reagents, ratios of mixtures, additives and thermal cycling conditions in order to obtain optimal results, these are listed in Table 3.3. The optimal combinations of primers and PCR conditions which supplied the data for this thesis are listed below:

Table 3.1 Previously published PCR Primers used on Kiwi DNA to generate MHC.

Primer Name	Source	MHC area	Primer Sequence
MHCABSF1	(Miller 2003)	Class IIB exon 2	CTGCACGCTCAGGGGTCTTCC A
MHCABSR2	(Miller 2003)	Class IIB exon 2	GAGGGGCTCCGGGGTCWCTG
Class1Ex3F	(Miller 2003)	Class I exon 3	CGAGTTWHYGGCTGTGAS
Class1Ex3R	(Miller 2003)	Class I exon 3	TCCRGGCASKYDTSCTYCA
325	(Edwards et al 1995a)	Class IIB exon 2	GTAGTTGTGNCKGCAGTANST GTCCAC
326	(Edwards et al 1995a)	Class IIB exon 2	GAGTGYCAYTAYYTNAAYGG YAC
M13 (F)	Invitrogen	Not applicable	TGTAAAACGACGGCCAGT
M13 (R)	Invitrogen	Not applicable	CAGGAAACAGCTATGACC

Table 3.2 PCR Primers designed during this Thesis for Kiwi MHC Class II B.

Primer Name	**Source**	**MHC area**	**Primer Sequence**
chMHCIIex1F *	Chicken data	Class IIB exon 1	CGTGCTGGTGGCACTGCT
chMHCIIex3R*	Chicken data	Class IIB exon 3	AGCACCACCAGCACCTGGT A
qMHCIIF	Quail data	Class IIB exon 2	AACGGCACCGAGCGSGTG AGG
qMHCIIR	Quail data	Class IIB exon 2	CTGAGGTGGACACRKWCT GCC
exon1aF	Chicken data	Class IIB exon 1	AGCACTGGTGGCGCTGTGA G
exon 3aR	Chicken data	Class IIB exon 3	TACGTCCAGTCCACGTTC
exon1bF	Chicken data	Class IIB exon 1	GGTCGTGCTGGTGGCACT
exon 3bR	Chicken data	Class IIB exon 3	CCAGCACCAACAGCAACT GG
kMIIint2R	Kiwi data	Class IIB intron 2	AGCCCTGCCAGATCCAGTG C
chMIIex 1b	Chicken data	Class IIB exon 1	GCTTGCGCTGTGAGCCCTG CC
chMIIex1c	Chicken data	Class IIB exon 1	GTGCTGGTTGCACTGCTGG C
kMIIex 3R	Kiwi data	Class IIB exon 3	ACCACGTGCTCCGTCTTCT CC
kMHCIIF	Kiwi data	Class IIB exon 2	AGCRGGTGAGGTWTTTG
kMHCIIR	Kiwi data	Class IIB exon 2	CTCAATGANGTCTGGC

Table 3.2 Continued PCR Primers designed during this Thesis for Kiwi MHC Class II B.

Primer Name	Source	MHC area	Primer Sequence
kmhcex1f	Kiwi data	Class IIB exon 1	CGGMAGTAGGTGTCCACA GA
kmhcint1a (F)	Kiwi data	Class IIB intron 1	GCCCATAGAAAGCAATGC CGT
kmhcint2R	Kiwi data	Class IIB intron 2	GTGTGGAGGGYGTGGGGG TGT
Boundint2	Kiwi data	Class IIB intron 2	CCAGCYCCRCCAGACYCA GGGC.
kMHCint2p (F)	Kiwi data	Class IIB intron 2	CTTCCACRRGGACAYGAGC
kmhcint2pR	Kiwi data	Class IIB intron 2	GCTCRTGTCCYYGTGGAAG
kmhcint1a	Kiwi data	Class IIB intron 1	GCCCATAGAAAGCAATGC CGT
kiwint2bR	Kiwi data	Class IIB intron 2	CAGCCCCGCCAGACCCAG GG
kiwex1F	Kiwi data	Class IIB exon 1	AGCCATGGGGACTGGTTG
kiwex1R	Kiwi data	Class IIB exon 1	TACTTGGCANBTGGYTCAC C
exonkiw1F	Kiwi data	Class IIB exon 1	CTGGAGCAGCGTTAGCAGT
exonkiw1R	Kiwi data	Class IIB exon 1	TGTCGGCCACRWRGAMCC
KiwintA1F	Kiwi data	Class IIB intron 1	CGTGACCTGCCTCTCTCTG
KiwintA2R	Kiwi data	Class IIB intron 2	AGGGCTCTCCYRTKCACT
KiwintB1F	Kiwi data	Class IIB intron 1	TGYNGTGTGGAGGGYGTG
KiwintB2R	Kiwi data	Class IIB intron 2	TKGGGAGCTGCACTCGAC

The early primers were designed by eye and later ones using Prime3 on line (Rozen and Skaletsky 2000). * Primers with asterisk were designed by Dr Leon Huynen. F denotes a forward primer and R is a reverse primer. Chicken and quail data was sourced from the NCBI database and used in primers design. Primers designed from kiwi data used sequences elicited by this project. All Primers were manufactured by Sigma Genesis.

Table 3.3 Range of PCR modifications used to optimise results.

1. The annealing temperatures used ranged from 45-65°C. Annealing temperatures are one of the most important parameters

2. The addition of BSA can enable binding of inhibitory complexes that may be present in the DNA source and inhibiting the PCR (Kreader 1996).

3. The addition of Betaine (~5%) can enhance DNA strand separation especially with GC rich templates (Henke et al 1997).

4. The addition of DMSO (range 1 – 10%) can enhance DNA strand separation and possible specificity and yield of PCR (Filichkin and Gelvin 1992).

5. The addition of formamide can increase the specificity and yield of PCR with GC rich templates (Sarkar et al 1990).

6. The addition of KCl may slow larger DNA denaturation so smaller DNA fragment production is favoured.

7. Taq - Varied concentration and types of Taq were tried; Platinum Taq showed the best response. Taq DNA Polymerase by Biolab gave a poor response when used with early degenerate primers. Roche Expand High Fidelity^{PLUS} PCR enzyme was used at one stage to reduce errors in amplifying DNA.

8. Variation in Thermal cycle programmes :

Number of cycles in thermal cycle programme – At the optimising stage minimising the number of cycles was used to decrease potential artefact production (Judo et al 1998; Zylstra et al 1998).

The range of cycles used varied from 25–40 cycles.

Several different "touchdown" programmes were used in the early stages of verifying if a particular combination of primers and reagents worked.

Variation in the time spent at different temperatures during the programme depending on the desired PCR product size.

9. The $MgCl_2$ concentrations ranged from 1.0 mM to 4.0 mM. It is crucial for the performance of DNA polymerase Taq. Increasing Mg^{2+} decreases the stringency of the reaction. Effective levels of Mg^{2+} can decrease due to forming complexes with dNTPs.

10. The pH of the buffer was varied from Invitrogen's PCR Buffer pH at 8.4 to occasionally a buffered PCR solution of pH 9 (The PCR buffer (at 10 x concentration) is: 500mM Tris-Cl, pH 8.8@ 20 C, 200mM (NH4)2SO4, and 10mg/ml BSA).

Table 3.3 continued Range of PCR modifications used to optimise results.

11. At the optimising stages "PCR plus 1" protocols were tried to reduce heteroduplex produced error (Thompson et al 2002). See 3.3.2.3 PCR conditions for 326/kmhcint2pr PCR plus 1.

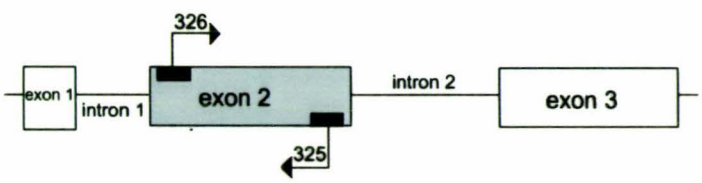
12. Changing the primer concentration (range 0.01 nM–0.05 nM) particularly helped when using degenerate primers. A higher concentration of degenerate primer proved very effective as the more degenerate it is the lower the relative concentration of effective primer present.

13. The dNTP concentration ranged from 200–300 µM. Too high a level of dNTP relative to Mg^{2+} concentration can inhibit a PCR reaction if Mg^{2+} is not also increased to compensate. Too low a level can also inhibit the PCR reaction as the dNTPs are needed to build the new DNA.

3.3.1 PCR using degenerate primers 325 & 326 for Class II B exon 2.

The optimised PCR Conditions for Primers 325 & 326 with Kiwi DNA were performed in 25 µl volumes containing Invitrogen PCR Reaction Buffer (final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl)), 2.5 mM $MgCl_2$, 200 µM dNTP, 0.5 units Invitrogen Platinum Taq, 1.6 µM of each primer and 1 µl of extracted feather DNA. PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler as follows: after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/58°C for 30 seconds/72°C for 30 seconds, for 30 cycles then finished with a final 30 second extension at 72°C. The PCR was then stored at 4°C overnight if it was not immediately processed.

Figure 3.1 The not to scale location of primers 325/326 in kiwi Class II B MHC.



A band ~210 bp in size was identified by Agar Gel Electrophoresis (AGE) in the PCR product (see section 3.4 Agar Gel Electrophoresis). The PCR product was then purified from unused reagents using a Sephadex Spin column (section 3.5.1 Sephadex Spin

columns). The purified DNA product from this was then inserted into a plasmid and transformed into bacteria to amplify target DNA using the Promega pGem®-T Easy Vector System (see section 3.6.1 pGem®-T Easy Vector System (Promega)). Suitable colonies of bacteria were picked and a PCR performed to amplify the DNA insert in the transformed plasmid using M13 primers (see section 3.3.6). The PCR product was then checked by AGE to ensure it was the expected size (see section 3.4 Agar Gel Electrophoresis). The M13 primer PCR product was then cleaned up by using ExoSAP (see section 3.11 ExoSAP) to remove ssDNA and unused dNTPs by digestion. The resulting DNA template was sequenced and analysed (see section 3.12.1 Sequencing with plasmid/PCR product.)

3.3.2 Amplification of Kiwi Class II B exon 2–3.

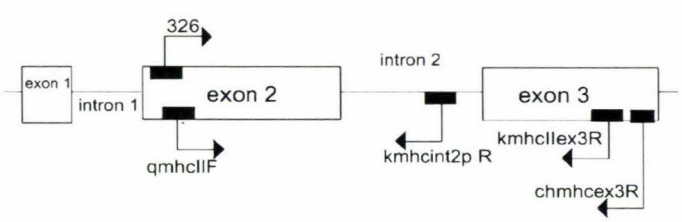
Successful Primers used:

qmhcll F/chmhcex3R

326/kmIIex3R

326/kmhcint2pr PCR plus 1

Figure 3.2 The not to scale location of primers for exon 2-3 in kiwi Class II B MHC.



3.3.2.1 PCR conditions for qmhcII F/chmhcex3R.

PCR Conditions for **qmhcII F/chmhcexR** primers were performed in a 20 µl volume containing Invitrogen PCR Reaction Buffer (final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 mM of each primer, and 0.5 units Proof-reading Taq (Roche Expand High Fidelity^{PLUS} PCR Enzyme mix) . The PCR amplification was conducted in a GeneAmp 9600 Thermal Cycler: after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/56°C for 30 seconds/72°C for 30 seconds, for 30 cycles then finished with a final 5 minute extension at 72°C.

The PCR product of this reaction, a ~800 bp band was then identified by AGE (see 3.4 Agar Gel Electrophoresis)and was removed from other bands by using Gelase and LM Agarose (see section 3.9.2 Gelase Technique). The DNA was purified by using Phenol: Chloroform (see section 3.2.3 Phenol: Chloroform Nucleic Acid Extraction) and then Sephadex Spin columns (see section 3.5.1 Sephadex Spin columns). The DNA was ligated and transformed using pGem®-T Easy Vector System (see section 3.6.1 pGem®-T Easy Vector System (Promega)). Minipreps were made of colonies with suitable sized inserts ~1000 bp, and DNA was extracted from the MiniPrep (see section 3.7.1 Minipreps). The DNA was sequenced with Big Dye Termination mix 3.1 (BDT 3.1) and the M13 Reverse primer for the ABI 3730 Genetic Analyzer (see section 3.12.1 Sequencing with plasmid/PCR product).

3.3.2.2 PCR conditions for 326/kmIIex3R.

PCR Conditions for **326/kmIIex3R** primers were performed in a 25 µl volume containing Invitrogen PCR Reaction Buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 1.6 mM of each primer, 1 M Betaine, 1.0 units Platinum Taq (Invitrogen) and 1 µl of DNA. The PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler, after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/50°C for 30 seconds/72°C for 60 seconds for 30 cycles, then finished with a final 5 minute extension at 72°C.

The PCR product of this reaction, a single ~700 bp band was identified by AGE (see section 3.4 Agar Gel Electrophoresis), and then the PCR product was cleaned in a S400 Sephadex Spin columns (see section 3.5.1 Sephadex Spin columns). The DNA was ligated and transformed using pGem®-T Easy Vector System (see section 3.6.1 pGem®-T Easy Vector System (Promega)). Minipreps were made of colonies with suitable sized inserts ~900 bp, and DNA were extracted from the MiniPrep (see section 3.7.1 Minipreps). The DNA was sequenced with Big Dye Termination mix 3.1 (BDT 3.1) and the M13 Reverse primer for the ABI 3730 Genetic Analyzer (see section 3.12.1 Sequencing with plasmid/PCR product).

3.3.2.3 PCR conditions for 326/kmhcint2pr PCR plus 1.

PCR Conditions for **326/kmhcint2pr** primers used a PCR plus 1 method (a second reconditioned PCR). Both stages of this PCR were performed in 25 µl volumes in a Hybaid Thermal Cycler with oil on top. The first PCR contained Invitrogen PCR Reaction Buffer (final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM dNTP, 1M Betaine, 0.8 mM of each primer, 0.5 units Platinum Taq (Invitrogen) and 1 µl of feather extract DNA . The PCR amplification was an initial step of 94°C for 2 minutes, and then the temperature was cycled 94°C for 30 seconds/55°C for 30 seconds/72°C for 60 seconds for 30 cycles. The second reconditioning PCR was a fresh PCR mixture of the same ingredients and concentrations as the first PCR, except instead of feather extract DNA, 1 µl of the first PCR's product was added. The new PCR was mixed and placed in the Hybaid for 94°C for 2 minutes, and then the temperature was cycled 94°C for 30 seconds/55°C for 30 seconds/72°C for 60 seconds, for 3 cycles.

The PCR product of this reaction, a single ~500 bp band, was identified by AGE (see section 3.4 Agar Gel Electrophoresis). The PCR product was purified by using phenol: chloroform (see section 3.2.3 Phenol: Chloroform Nucleic Acid Extraction) and then passed through a S400 Sephadex Spin columns (see 3.5.1 Sephadex Spin columns). The DNA was ligated and transformed using pGem®-T Easy Vector System (see section 3.6.1 pGem®-T Easy Vector System (Promega)). Minipreps were made of colonies with suitable sized inserts (~700 bp), and DNA was extracted from the Minipreps (see section 3.7.1 Minipreps). The DNA was sequenced with Big Dye Termination mix 3.1 (BDT 3.1) and the M13 Reverse primer on the ABI 3730 Genetic Analyzer (see section 3.12.1 Sequencing with plasmid/PCR product).

3.3.3 5′ RACE PCR

Invitrogen's 5′ RACE System for Rapid Amplification of cDNA Ends Kit was used as per manufacturers' instructions. Invitrogen Superscript III Reverse Transcriptase was used to synthesis cDNA from the mRNA. The product was purified by the Qiagen QIAquick PCR Purification Kit method using a microcentrifuge as described in the manufacturers' instructions. The purified cDNA was then given a Poly C tail using Invitrogen Terminal transferase (TdT) and dCTP as described in the manufacturers'

instructions. A PCR was used to amplify the dC-tailed cDNA using the kit's Abridged Anchor Primer (5' AAP) and a gene specific primer (RACE-2 / GSP 3-2) that was designed from the kiwi sequences generated using primers 325/326, i.e., internal to Class II B exon 2. A second amplifying PCR was then performed using a template from the first PCR and the Abridged Universal Amplification Primer (AUAP) with another kiwi specific primer (RACE-3 / GSP 3-3). Two sets of the two gene-specific primers were designed by eye from Kiwi Class II B exon 2 sequences obtained earlier in this project for the gene specific primers. See Fig 3.3 for relative primer positions. The primer sequences were:

RACE – 1 GTCTGKCWGGCYGTTCCAGTACTTGGCA

RACE – 2 TGGYTCACCCAGGGGGCTGTCGGCCAC

RACE – 2 was used in first amplifying reaction with 5′ AAP.

RACE – 3 ACGTCACTRTCGAAGTGCACSWMCTGCTGC

RACE – 3 was used in second amplifying reaction with AUAP.

GSP-3-1 TCGCCGTTCTGGATCACGTCCGTGGCCACC

GSP-3-2 ACGCGCTCCGTCTCCTCCCGCCCGTTCTTG

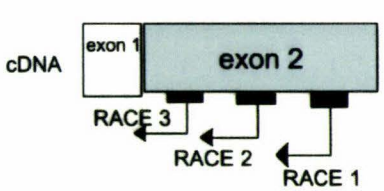
GSP-3-2 was used in first amplifying reaction with 5′ AAP.

GSP-3-3 CCCGTCACGTAGCAGAGCAGCCTGTCCG

GSP-3-3 was used in second amplifying reaction with AUAP.

The series of PCR reactions with RACE 2 & 3 produced a strong ~350 bp band, on 1.2% MS Agarose after AGE. The series of PCR reactions with GSP 3-2 & 3-3 produced a very weak band and this product was not pursued in favour of the RACE primers product.

Figure 3.3 Position of kiwi specific primers in a 5′ RACE PCR.(not to scale)



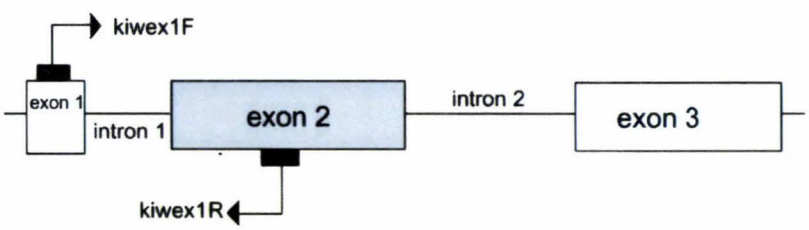
The 5′ RACE PCR product was cloned with Invitrogen One Shot® MAX Efficiency® DH5α™-T1R Kit, (see section 3.6.2 Invitrogen One Shot® MAX Efficiency® DH5α™-T1R Kit). The colonies were checked for inserts and DNA was extracted by using Invitrogen PureLink™ Quick Plasmid MiniPrep Kit, (see section 3.7.2 Invitrogen

PureLink™ Quick Plasmid MiniPrep Kit). The DNA was sequenced by BDT 3.1 sequencing reaction and analyzed in an ABI 3730 Genetic Analyzer (see section 3.12.1 Sequencing with plasmid/PCR product).

3.3.4 PCR Conditions for kiwex1F/kiwex1R .

PCR for primers **kiwex1F/kiwex1R** was performed in a 25 µl volume containing Invitrogen PCR Reaction Buffer (final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 1.5mM $MgCl_2$, 200 µM dNTP, 0.8 mM of each primer, 1M Betaine, 0.5 units Platinum Taq (Invitrogen) and 1 µl of DNA . The PCR amplification was carried out in a Hybaid Thermal Cycler so an oil layer was placed over the PCR mix before heating. After an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/55°C for 30 seconds/72°C for 60 seconds for 30 cycles, and then finished with a final 20 minute extension at 72°C. See Fig 3.4 for the positions of the primers.

Figure 3.4 The position of primers for exon 1-2 in kiwi Class II B MHC (not to scale).



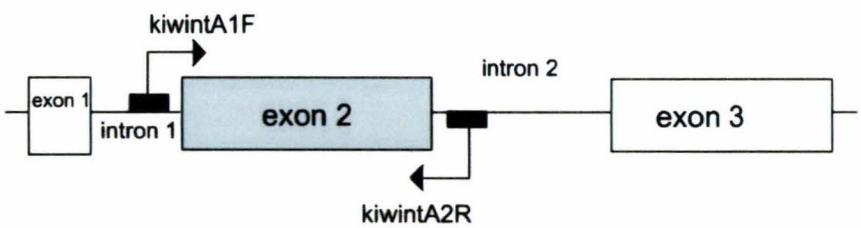
This PCR produces two bands ~700 bp band and ~300 bp band on AGE (see section 3.4 Agar Gel Electrophoresis). The 700 bp band was extracted using QIAEX II Gel Extraction System (see section 3.9.1 QIAEX II Agarose Gel Extraction.). The DNA was then cloned by TOPO TA cloning (see section 3.6.3 Invitrogen TOPO TA Cloning). Minipreps were made of colonies with suitable sized inserts ~900 bp, and DNA was extracted from the MiniPrep (see section 3.7.1 Minipreps). The DNA was sequenced using the Big Dye Termination mix 3.1 (BDT 3.1) and M13 Reverse primer and read in the ABI 3730 Genetic Analyzer (see section 3.12.1 Sequencing with plasmid/PCR product).

3.3.5 PCR conditions for KiwintA1F & KiwintA2R on Kiwi DNA.

PCR for primers **KiwintA1F/KiwintA2R** was performed in a 25 µl volume containing PCR Reaction Buffer ((final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2.0

mM $MgCl_2$, 160 µM dNTP, 0.8 mM of each primer, 1 M Betaine, 0.5 units Platinum Taq (Invitrogen) and 1 µl of feather extract DNA. The PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/60°C for 30 seconds/72°C for 60 seconds for 30 cycles, then finished with a final 10 minute extension at 72°C. Fig 3.5 shows the relative position of the primers.

Figure 3.5 The position of primers KiwintA1F & KiwintA2R in kiwi Class II B MHC (not to scale).



The PCR product was ligated with pCR 2.1® Vector (See section 3.6.4 Invitrogen pCR 2.1® Vector) and transformed into SURE®2 Supercompetent cells (see section 3.6.5 Stratagene SURE®2 Supercompetent cells). The resulting plasmids were amplified with TempliPhi™ (see section 3.10 TempliPhi™) and then sequenced (see section 3.12.2 Sequencing TempliPhi product) with Big Dye Termination mix 3.1 (BDT 3.1) and the M13 Reverse primer for analysis in the ABI 3730 Genetic Analyzer.

3.3.6 PCR for plasmids with M13 Primer Sites.

A 96 well plate was used to contain the PCR products which were in 10µl volumes. Each contained PCR Reaction Buffer (final concentration 20 mM Tris-HCl (pH 8.4), 50 mM KCl), 40ng of M13 forward and reverse primer, 2.5 mM $MgCl_2$, 0.01 mg Bovine Serum Albumin (BSA), 10µM dNTP and 0.25 units Platinum Taq (Invitrogen). A sterilised 10µl pipette tip was touched to the bacterial colony being checked and then placed in the PCR mixture and twirled for ~ 5 seconds. A fresh sample of the colony was also touched to another labelled LB Agar plate with Ampicillin (100µg/ml) and incubated overnight at 37°C. The PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 20 seconds/54°C for 20 seconds/72°C for 20 seconds for 35 cycles, then finished. To check the size of the inserts a sample of the PCR was run on an AGE.

A plasmid of the correct size DNA insert shows a PCR product ~ 200 bp larger than the original size of the DNA inserted, as some of the plasmid is also amplified attached to the insert when using the M13 primers.

3.4 Agar Gel Electrophoresis (AGE).

This AGE protocol was used to identify bands which varied in size from ~ 200 bp to ~1000 bp. Samples ~2µl of PCR product mixed 75:25 with loading dye mixture was placed into separate wells in a slab made from 1% MS Agarose/1% LM Agarose in TBE solution [x1.0]. It was run for 20 minutes at 100mV in a TBE solution [x0.5] and then soaked for 10 minutes in dilute Ethidium Bromide solution. The gel slab was viewed under UV light to compare the bands formed in the PCR against an Invitrogen 1kb plus DNA ladder.

The loading dye was a mixture of bromophenol blue and xylene cyanol in a 40% (w/v) sucrose solution and 10mM Tris pH 7.5 then stored at 4°C. The tracking dyes (bromophenol blue and xylene cyanol) were added as powders to give the colour intensity.

3.5 PCR purification techniques

3.5.1 Sephadex Spin columns

Lab-made Sephadex spin columns were used to separate out smaller molecules such as primers and dye from the larger DNA template in PCR products by gel filtration (Sephadex beads). The matrix acts as a molecular sieve, smaller molecules entering and leaving the matrix pores, thereby traversing the length of the column relatively slowly compared to larger molecules, which can not enter the gel pores and therefore elute with the void volume.

A 200µl plastic disposable pipette tip with a filter tip at the distal end was packed with S200 or S400 Sephadex beads, by aliquoting in the beads and centrifuging for 1 minute at RCF = 840g. Prior to centrifuging the tip was wedged into a hole cut in the lid of an Eppendorf tube to hold it in place as it spun, and simultaneously allowing the waste fluid to drain into the bottom of the Eppendorf tube. The spin column is now ready to use and inserted into a fresh clean labelled Eppendorf tube and the PCR product (20-60

µl) is applied to the wide top (proximal end) of the tip, and then centrifuged for 1 minute at RCF = 840g. The resulting fluid contains the larger DNA molecules.

3.5.2 QIAquick PCR Purification Kit Protocol using a microcentrifuge (Qiagen)

The QIAquick PCR Purification Protocol with a microcentrifuge was used as per manufacturers instructions in their handbook (ver. July 2002), to produce pure DNA from the PCR product mixture.

3.6 Cloning techniques

3.6.1 pGem®-T Easy Vector System (Promega)

DNA was ligated and transformed using the Promega pGem®-T Easy Vector System as per manufacturers' instructions, Technical manual 042 (revised 3/03).

3.6.1.1 Tailing of blunt-ended DNA.

Blunt ended DNA was given an –A tail before being ligated. This reaction was performed as per manufactures instructions in Promega pGem®-T Easy Vector System Technical Manual No 42 (revised 3/03). DNA amplified in PCRs with Platinum Taq (Invitrogen) already have an A overhang so do not require this step.

3.6.1.2 Ligation of DNA template to pGem®-T Easy Vector

The DNA template was ligated into the pGem®-T Easy Vector as per the manufacturers instructions, Technical manual 042 (revised 3/03), using the 2x Ligation buffer and incubating at 4°C overnight, for maximum transformants.

3.6.1.3 Transformation of ligated pGem®-T Easy Vector into the JM109 High Efficiency Competent Cells

The DNA ligated to the pGem®-T Easy Vector was and transformed into the JM109 High Efficiency Competent Cells as per manufacturers instructions. The bacteria was grown overnight at 37°C on LB Agar with Ampicillin/X-Gal. Suitable colonies (white ones) were checked for inserts using a standard PCR with M13 forward and reverse primers (see section 3.3.6 PCR for plasmids with M13 Primer Sites) and then checked by AGE for band size (see section 3.4 Agar Gel Electrophoresis). Colonies with

suitable inserts were then cultivated and purified in a MiniPrep (see section 3.7 MiniPrep) to produce a suitable amount of DNA for sequencing.

3.6.2 Invitrogen One Shot® MAX Efficiency® DH5α™-T1R Kit.

The Chemically Competent Cells supplied in the Kit were stored and used as per manufacturer's instructions. The colonies were grown on LB Agar with Ampicillin and X-Gal (see method 3.13.2) at 37°C overnight.

3.6.3 Invitrogen TOPO TA Cloning

The reaction was performed using the stock salt solution as per the manufacturer's instructions and the resulting plasmids were transformed into chemically competent Invitrogen DH5α™-T1R cells (see section 3.6.2). The Invitrogen TOPO TA Cloning process uses topoisomerase bound to the vector (pCR®4-TOPO®) to join the PCR product DNA with an A overhang onto the vector plasmid at the T- overhanging ends. The TOPO TA cloning was performed on PCR products with a final extension time 20 minutes when in the Thermal Cycler to ensure the maximum number had "A" tails to join to the overhanging T-.

3.6.4 Invitrogen pCR 2.1® Vector

The Invitrogen pCR 2.1 vector was ligated to the PCR product DNA using T4 DNA ligase as per manufactures instructions in the Invitrogen TA Cloning® Kit manual (ver. W, Feb. 2006).

3.6.5 Stratagene SURE®2 Supercompetent cells.

SURE®2 Supercompetent cells (Stop Unwanted Rearrangement Events) have been modified so they lack components in pathways that perform rearrangements and deletion of non standard secondary and tertiary DNA structures. This means they are less likely create artefacts by random recombination events in the DNA they were amplifying. The Transformation protocol used was as per manufacturer's instructions, and the colonies were grown on LB Agar with Ampicillin and X-Gal (see section 13.2) at 37°C overnight.

3.7. MiniPrep Techniques

3.7.1 Minipreps as per (Jones and Schofield 1990).

This method of alkaline lysis for isolating plasmid DNA was used as per the authors' instructions, except at the final stage instead of drying under reduced pressure the supernatant DNA pellet was air dried.

3.7.2 Invitrogen PureLink™ Quick Plasmid MiniPrep Kit.

Plasmids were isolated from overnight cultures made in LB broth at 37 °C, following the manufacturers instructions provided in the Invitrogen PureLink™ Quick Plasmid MiniPrep Kit for purification using centrifugation. The resulting purified DNA was stored at 4°C and used within 24 hours for sequencing. DNA quantification was performed by Nanodrop technique (see section 3.8)

3.7.3 QIAprep Spin MiniPrep Kit (Qiagen)

Plasmids were isolated from overnight cultures made in LB broth at 37 °C, following the manufacturers instructions provided in the QIAprep Spin MiniPrep Kit for Plasmid DNA Purification Using the QIAprep Spin MiniPrep Kit and a Microcentrifuge. The resulting purified DNA was stored at 4°C and used within 24 hours for sequencing. DNA quantification was performed by nanodrop technique (see section 3.8).

3.8. Nanodrop ND-1000 Spectrophotometer

The concentration of DNA in a solution was estimated, as per manufacturers' instructions, using a nanodrop ND-1000 Spectrophotometer (Biolab Scientific).

3.9. DNA extraction from Agarose Gel

3.9.1 QIAEX II Agarose Gel Extraction (Qiagen)

A 2% LM Agarose gel in chilled (4°C) TALE buffer was run at 80 mV. The DNA band was cut from the gel under UV light using a clean straight-edged razor. The DNA was extracted from the gel using the QIAEX II Gel Extraction Kit and protocol in the QIAEX II Handbook for DNA Extraction from Agarose Gels (ver. Feb 1999).

3.9.2 Gelase technique (Epicentre)

DNA amplified in PCR's and run in AGE gels were extracted from the AGE gel by using Epicentre Gelase™. The agarose gel-digesting preparation was used as per manufacturers' instructions.

3.10 TempliPhi™ (Amersham).

TempliPhi™ kits (Amersham Biosciences, Piscataway, NJ) uses Φ29 DNA polymerase for a rolling amplification of circular DNA at a constant temperature (Dean et al 2001; Nelson et al 2002). TempliPhi™ produces consistent quality and quantity of DNA templates for DNA sequencing. The amplification method was performed isothermally at 30 °C, and generated an estimated 107-fold amplification in 4 – 6 hr. A large amount of product can be generated from DNA from purified plasmids, bacterial cells, or small amounts of saturated cultures. Phi29 (Φ29) DNA polymerase has proofreading activity, generating templates that can be used directly in sequencing reactions. A TempliPhi DNA Amplification Kit from Amersham Biosciences (currently GE Healthcare) was used, but the protocol was successfully modified to use lower quantities of reagents per reaction.

All the TempliPhi components were kept on ice prior to use. A fresh colony was placed in 100µl of milliQ water and gently vortexed. 2.5 µl of TempliPhi sample buffer was placed in each well of the 96 well plate and 1 µl of the colony/milliQ water mix was also added. The bacteria was denatured by heating at 95°C for 3 min, then the plate was centrifuged (1 minute at RCF = 840g) and placed on ice. The TempliPhi reaction involved adding 2.5 µl TempliPhi Reaction Buffer and 0.1 µl TempliPhi Enzyme to the plate well and mixing, then incubating at 30°C for 4-18 hours. The reaction was then stopped by heating to 65°C for 10 min, and placing on ice. The 96 well plates were then covered with an airtight non reactive cover and if they were not sequenced immediately, stored at -20°C.

3.11 ExoSAP

PCR product was cleaned immediately prior to sequencing using ExoSAP to digest ssDNA and unused dNTPs. ExoSAP is Shrimp Alkaline Phosphatase (SAP) (1U/µL) (Roche Cat. No. 1 758 250) and Exonuclease I (Exo)(10U/µL) (New England Biolabs Cat. No. M0293S), they are stored at -20°C until needed. To make up 60µl of the mixture add 2 µl Exonuclease I (20 units), 6µl Shrimp Alkaline Phosphatase (6 units) and 52µl dH_2O. To each well of 96 well plate containing 10µl DNA template add 2µl of the ExoSAP mixture. Place in GeneAmp 9600 Thermal Cycler for 37°C for 15 minutes and 80°C for 10 minutes. The resulting mixture is then immediately sequenced.

3.12. Sequencing.

3.12.1 Sequencing with plasmid/PCR product.

Sequencing was performed using Big Dye Termination mix 3.1 (BDT 3.1; Applied Biosystems) with the M13 Reverse primer. A 10µl reaction was used with 1 µl of BDT 3.1 dye, 2µl of BDT 3.1 Buffer, 3.2pmol M13 Reverse primer, and 150-300 ng of plasmid template or 3-10ng PCR DNA product. The reaction was incubated in GeneAmp 9600 at 96°C for 1 minute, then for 30 cycles of 96 °C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, and store at 4°C. Prior to analysing, the Agencourt CleanSEQ Sequencing Reaction Clean-Up system was used as per manufacturer's instructions to remove unincorporated dyes, nucleotides, salts and contaminants. It was analysed in a ABI 3730 Genetic Analyzer by the Allan Wilson Centre Genome Service at Massey University in Auckland, as per manufacturers instructions.

3.12.2 Sequencing TempliPhi product.

Sequencing was performed using Big Dye Termination mix 3.1 (BDT 3.1) with the M13 Reverse primer. A 20µl reaction contained 2µl of BDT 3.1 dye, 4µl of BDT 3.1 Buffer, 3.2pmol M13 Reverse primer, and 1 µl of 1:10 dilution of TempliPhi product. The reaction was incubated in GeneAmp 9600 thermal cycler at 96°C for 1 minute, then for 30 cycles of 96 °C for 10 seconds, 50°C for 15 seconds, and 60°C for 1 minute, and store at -20°C until sequenced in a ABI 3730 Genetic Analyzer, as per manufacturers instruction. Prior to analysing, the Agencourt CleanSEQ Sequencing Reaction Clean-Up system was used as per manufacturers' instructions to remove unincorporated dyes, nucleotides, salts and contaminants.

3.13. Agar Plates

3.13.1 LB Agar plate with Ampicillin (100µg/ml)

The LB Agar plates with Ampicillin were made as per instructions in Promega pGem®-T Easy Vector System Technical Manual No 42, Appendix B.

3.13.2 LB Agar plate with Ampicillin/X-Gal

The LB Agar plates with Ampicillin/IPTG/X-Gal were made as per instructions in Promega pGem®-T Easy Vector System Technical Manual No 42 Appendix B, except no IPTG was added to the mixture.

Chapter 4

The Process of designing PCR primers for the amplification of the Major Histocompatibility Complex (MHC) of the Kiwi (*Apteryx spp.*)

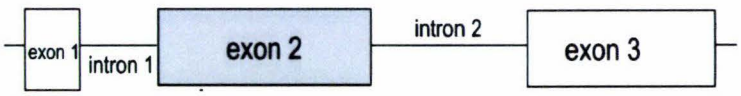
Introduction

This chapter records the logical progression of this thesis from the start until the point where a section of kiwi MHC coding for the Class II B Protein Binding Region (PBR) could be reliably amplified, separated and sequenced from a NIB kiwi. A summary of the individual steps is available as a flowchart, Fig 4.1. At the start we knew of no primers for a PCR that worked on any ratite MHC, let alone kiwi. So this thesis began by testing a variety of degenerate avian MHC primers under a range of PCR conditions. Eventually a pair of degenerate primers worked on kiwi DNA under modified conditions. The resulting sequences due to their similarity are believed to belong within the MHC Class II B exon 2, i.e., the Protein Binding Region (PBR) of the Class II receptor. To enable better analysis it was necessary to design primers that are positioned either side of exon 2 i.e., outside of exon 2 and inside intron 1 and intron 2. More primers were designed and PCR protocols optimised to produce DNA sequences from exon 2 to exon 3, thus sequencing intron 2. Exon 3 tends to be conserved so primers were designed from sequences of Class II B exon 3 of chickens and quail deposited the NCBI database (Table 3.2). To obtain the sequence from exon 1 to 2 a 5′ RACE PCR was performed on cDNA. New primers and PCR protocols were then designed to sequence from exon 1 to exon 2. The resulting sequences when placed in Sequencher™ 4.6 software and showed by contiguous overlap of the DNA that exons 1–3 are consistent with a DRB-like Class II B MHC when compared to similar sequences on the NCBI database.

Figure 4.1 Flowchart of the stages of this project to sequence Kiwi MHC.

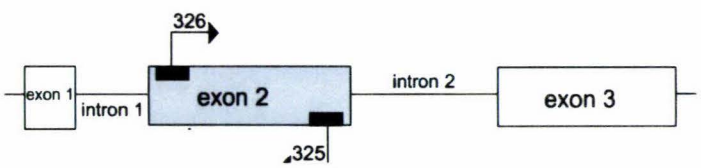
Step 1

Optimise a PCR using published degenerate primers for avian MHC to work on Kiwi DNA.



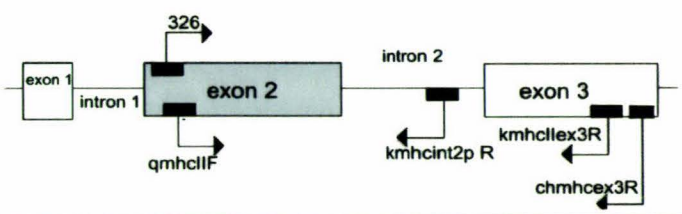
Step 2

Sequence Kiwi MHC made using primers 325 & 326 (Edwards et al. 1995). The sequence is an internal segment of Class II B exon 2 MHC.



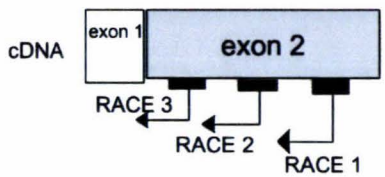
Step 3

Design Kiwi Class II MHC primers to extend sequencing from Class II B exon 2 to exon 3.



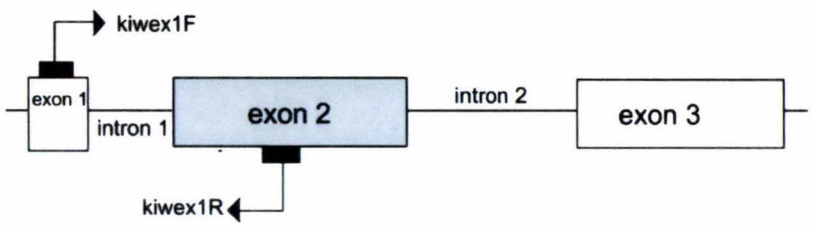
Step 4

Use a 5′ RACE PCR to sequence Kiwi Class II B, exon 1 and exon 2.



Step 5

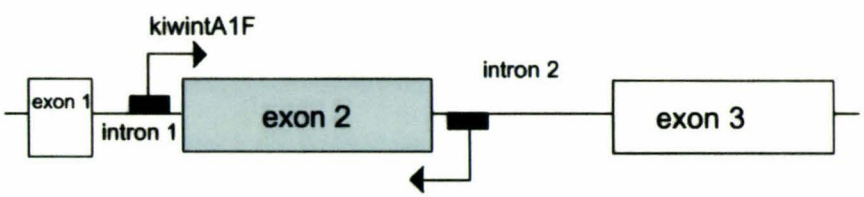
Design Kiwi Class II MHC primers **(kiwex1F/kiwex1R)** to sequence from Class II B exon 1 to exon 2.



Step 6.

Design primers that are sited in the introns either side of Kiwi Class II B exon 2, to sequence the entire exon.

Optimise the process from PCR to sequencing of Kiwi Class II B exon 2.



Step 7.

Use **kiwintA1F & kiwintA2R** to sequence the Class II B exon 2 (PBR) of three different Kiwi species and assess the level of polymorphism in each

The nomenculture for the Human MHC (HLA) refers to a particular Class II loci as DR, and its β strand as DRB. Using the Kiwi MHC sequences, primers were designed for the introns near the exon 2 boundaries, and a PCR optimised for their use. A diagram of the successful primers and their relative positions is available in Fig 4.2. The final primers, KiwintA1F & KiwintA2R were then used on 3 separate populations of Kiwi to evaluate the amount of MHC polymorphism of each population. The results of this final step are presented in Chapter 5.

Other investigations into the MHC Class II B and especially DRB-like areas have shown it is easy to produce errors and artefacts, during any stage from DNA extraction through PCR to sequencing (Edwards et al 1995a; Kennedy et al 2002; Weber et al 2004). The series of approaches taken to minimise errors affecting the results is reviewed in the conclusion of this chapter.

Step 1 Optimise a PCR using published degenerate primers for avian MHC to work on Kiwi DNA.

Being unable to find any primers for the MHC of the *Apteryx* genus or another ratite, it was necessary to start with degenerate primers used in Neognathae. Initially this was pursued using degenerate class I and class II primers from published articles and designed from known sequences on the NCBI database (Benson et al 2004). The primers were made by Sigma Genosys and the PCR conditions modified in a variety of ways to optimise the desired product. Table 3.1 lists the initial avian primers used which had been previously designed for MHC in Neognathaes not Paleognathaes. The best response by Kiwi MHC DNA was to the degenerate primers 325 & 326, designed by Edwards et al. (Edwards et al 1995a). These primers are internal to exon 2 of Class II B. A PCR was optimised using Kiwi DNA to minimise non-pertinent product and maximise yield of the 210 bp band.

It was necessary throughout this project to optimise a series of PCR reactions with a variety of primers. Table 3.3 lists the various modifications used in developing different

PCR conditions during this thesis e.g., the addition of reagents like betaine. Only the finalised PCR conditions for each set of primers are recorded in Chapter 3.

Step 2 Sequence Kiwi MHC made using primers 325 & 326. The sequence is an internal segment of Class II B exon 2 MHC.

An optimised PCR for primers 325 & 326 with Kiwi DNA was performed and processed to obtain a DNA sequence. The processing methods used from PCR to sequencing are described in chapter 3 section 3.3.1.

A 210 bp long section was sequenced, and trimmed to 160 bp when the primers are removed from each end. Primer sites were not included in the analysis of the sequences since these reflect the primer sequence and not necessarily the original DNA sequences.

The sequences were compared to others available on the NCBI database by use of the BLAST software (Wheeler et al 2007). It showed a high similarity to avian class II B exon 2 sequences i.e., the PBR region of the class II MHC. This was also confirmed by checking for Conserved Domains against the NCBI database which showed a significant alignment with MHC Class II B (Geer et al 2002; Marchler-Bauer and Bryant 2004). More specifically this DNA sequence was similar to DRB type sequences which is a commonly found type of Class II MHC.

DNA sequencing is regarded as the "gold standard" in studies of MHC polymorphism (Knapp 2005a), and is the method pursued in this project. Other techniques can be used e.g., SSCP (Single Strand Conformational Polymorphism) and DGGE (Denaturing Gradient Gel Electrophoresis) to separate out the different alleles generated in the PCR. Both these techniques were considered and after an initial attempt to separate out the different sequences produced by an individual using a SSCP it was abandoned in order to concentrate resources on the DNA sequencing approach. However for a large project with a large number of samples these other techniques could be more cost effective.

Step 3 Design Kiwi Class II primers to extend sequencing from Class II B exon 2 to exon 3.

The nucleotide sequence of kiwi MHC generated using the primers 325 & 326 in combination with sequences of avian MHC in the NCBI database (Benson et al 2004) was used to design primers to span from exon 2 to exon 3. Chicken data and quail data deposited in NCBI was used for two reasons. One, there was a lot more chicken data available compared to other birds at the time and two, the galliformes are thought to be basal in Neognathae and closer to ratites than other Neognathae (Sibley and Ahlquist 1990). The list of primers used and the steps taken to optimise PCRs are reported in Tables 3.2 & 3.3. New degenerate primers designed across conserved regions from an alignment of exon 3 sequences proved successful. Unfortunately this approach did not work for designing successful primers to exon 1, so another approach was used.

A series of three different primer pairs and PCR reactions was used to obtain the sequence of kiwi DNA from exon 2 to exon 3. The successful primers/PCR conditions and the processing used to sequence the results are listed in chapter 3. The conditions for qmhcllF/chmhcex3R are in section 3.3.2.1: 326/kmIIex3R.in section 3.3.2.2; and 326/kmhcint2pr in section 3.3.2.3. kmIIex3R and kmhcint2pr were designed from kiwi sequences produced by the quail and chicken primers qmhcIIF & chmhcex3R.

The data from the reactions was assembled with data generated in step 4 & 5 to show a continuous DNA sequence from exon 1 to exon 3 by using contiguous overlapping sequences. It can be seen in Appendix A Table A.1. As expected the DNA sequence for exon 3 was very similar to other avian exon 3. The intron 2 DNA sequence was not very similar to other Class II B intron 2 sequences in the NCBI database except for a small 65 bp segment that showed a high similarity (80-85%) with a segment of Class II B intron 2 reported in Humboldt penguins *(Spheniscus humboldti)* and several birds of prey (Kikkawa et al 2005; Alcaide et al 2007). (See Table A.2)

Step 4 Use a 5′ RACE PCR to sequence Kiwi Class II B, exon 1 and exon 2.

A 5′ RACE PCR was used on cDNA (complementary DNA) to provide information on exon 1 by sequencing from exon 2 directly to exon 1. The cDNA was generated from mRNA and it does not contain the intron 1 sequence between exons 1 & 2.

The description of the 5′ RACE PCR method used and the processing of the DNA until sequencing are given in Chapter 3 section 3.3.3. The data contributed to the map spanning from exon 1 to 3 in Appendix A. The sequences generated probably contain the whole of exon 1, as the starting point is consistent with a translation initiation point called a Kozak sequence (Kozak 1996). Prior to using the 5′ RACE PCR several unsuccessful attempts were made using Blunt hairpin gene walking and linked hairpin primer gene walking, to obtain DNA sequences for exon 1 to exon 2.

Step 5 Design Kiwi Class II MHC primers kiwex1F/kiwex1R to sequence from Class II B exon 1 to exon 2.

Primers kiwex1F & kiwex1R were designed to span exon 1 to exon 2 using the results of steps 2 & 4. The PCR conditions optimised for kiwex1F/kiwex1R and the processing to sequencing are recorded in Chapter 3 section 3.3.4.

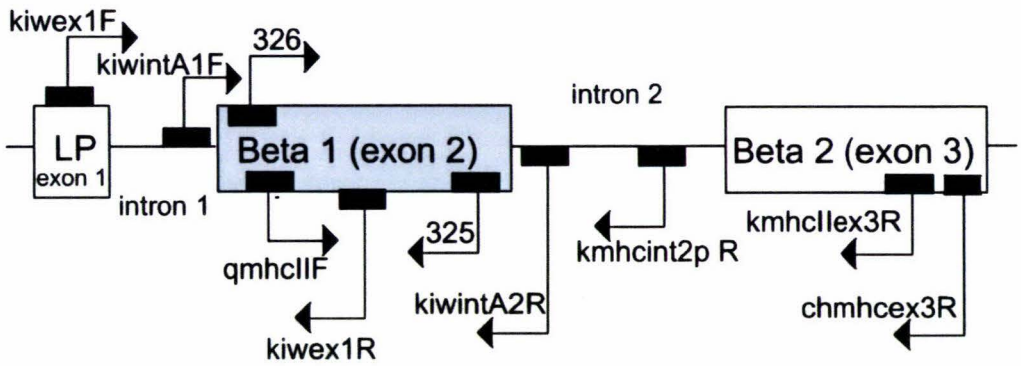
Step 6 Design primers that are sited in the introns either side of Kiwi Class II B exon 2, to sequence the entire exon & Optimise the process from PCR to sequencing of Kiwi Class II B exon 2.

Using the MHC Class II B Kiwi DNA sequence elicited by this project, primers KiwintA1F & KiwintA2R were designed and optimised to sequences Kiwi DNA from the intron 1 to intron 2. The optimised conditions for this PCR are recorded in chapter 3 section 3.3.5. Figure 4.2 shows the position of primers developed in this thesis.

Step 7 Use kiwintA1F & kiwintA2R on Three kiwi populations.

Use kiwintA1F & kiwintA2R to sequence the Class II B exon 2 (PBR) of three different Kiwi species and assess the level of polymorphism in each population.
The results of this final step are recorded in chapter 5.

Figure 4.2 Diagram of primer positions on Kiwi MHC Class II B exons 1- 3 (not to scale).



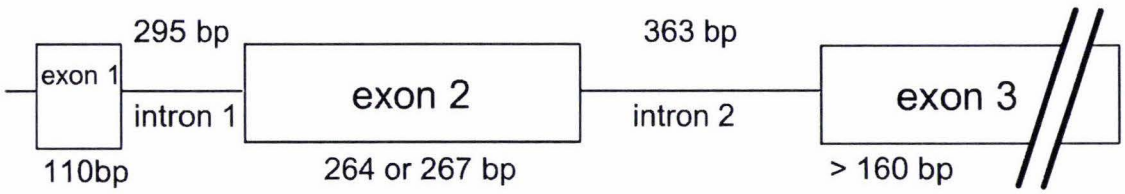
The arrow indicates the direction of the primer.

Discussion

The process described here has yielded the first paleognathae MHC gene ever sequenced of which I am aware. This provides a solid basis from which to further explore kiwi and other ratite MHC systems. In this chapter an overview of the processes involved in getting to the position of being able to sequence DRB like Class II MHC of the kiwi is given. The results and their implications to Kiwi conservation and our understanding of avian MHC evolution are presented more fully in Chapter 5.

In Appendix A, DNA is assembled from the various steps in the project to create a sequence from exon 1 to exon 3. The overview of the sizes of the introns and exons sequenced is shown in Fig. 4.3. The assembled contigs suggest that the size of introns and exons of DRB like Class II MHC in Kiwi is moderately different to that published in other birds. The putative length of intron 2 (363bp) in NIB Kiwi is greater than seen in birds of prey (250-280 bp) but smaller than those seen in passerines (380 -1460 bp) (Edwards et al 1998; Hess et al 2000; Gasper et al 2001; Alcaide et al 2007). Passerines and birds of prey (except Falco) have shown an intron 1 of approx. 440bp which is longer than the 295bp of the Kiwi (NIB). Falco species have shown an even longer (1-1.5kb) intron 1 (Alcaide et al 2007).

Figure 4.3 The putative length of introns and exons of NIB Kiwi in Class II B MHC.



This is an appropriate point in the thesis to discuss the technical difficulties that were overcome, and in particular how the minimisation of artefact production was achieved. Errors producing artefacts can occur at any and all stages of processing the DNA from the sample to sequencing and a variety of techniques have been used to deal with this problem. The production of artefacts during PCR and cloning of the avian MHC has been quantified as: Point mutations occurring in 1 per 1,000 nucleotides and recombination events occurring in about 1 in 20 templates (Edwards et al 1998). Jansen and Ledley (1990) suggested in vitro recombination events could affect ¼ of cloned PCR fragments from heterozygotes possibly due to excision repair of heteroduplexes during cloning. Various methods have been suggested to reduce the formation of these recombination events. The following is a discussion on how they have been dealt with in this project, which culminated in the final technique applied to three populations of Kiwi in chapter 5.

Sample quality is important to produce adequate amounts of high molecular weight DNA. Although non invasive samples have a great attraction in conservation research due to the relative ease of obtaining them, concerns have been expressed about the quality of such samples used for analysis (Piggott and Taylor 2003). This concern is stronger in regard to faecal samples than feather samples (Knapp 2005b). The technique used here for DNA extraction from feathers has been reliably used for other nuclear DNA testing purposes e.g., the sexing of ratites (Huynen et al 2002). Most feathers produced enough DNA templates for the PCR reaction, those that did not tended to be the feathers with no visible follicle tissue attached at the base.

The final number of PCR cycles was kept to thirty or less to reduce the number and complexity of artefacts which increases with the number of amplification cycle (Zylstra et al 1998). High levels of amplifying cycles may in the later cycles produce an abundance of template and a shortage of primers leading to incorrect priming. Another possibility is the polymerase enzyme has more opportunity to "jump" from one template to another before completing a strand's synthesis (Judo et al 1998). In vitro recombination events are thought to produce 1 -5 % of variant sequences (Jansen and Ledley 1990). The recombination is due to template switching or jumping, when the incomplete amplified sequences of one allele joins with the template of another allele, the result is a chimera with bits of two different allele sequences.

To minimise the formation of heteroduplexes prior to cloning a "Reconditioning PCR" (PCR plus one) was used with the primers 326/kmhcint2pr. After the first 30 cycle PCR, a second, three cycle PCR followed using a small amount of the first PCR as template. This process is thought to minimise the production of heteroduplex molecules before the cloning step, which may later be mistaken for true sequences (Thompson et al 2002). It did appear to be beneficial i.e., less artefact was produced but it was not significantly better. No direct comparison, however, was done comparing the results of a reconditioned PCR with a normal PCR in this project.

During this project three types of Taq were used: Invitrogen Platinum Taq, Biolab Taq DNA Polymerase and Roche Proofreading Taq. The Biolab Taq DNA Polymerase was unable to amplify product with the 325/326 primers, and was not used again. The Invitrogen Platinum Taq was found to perform well at all stages of its use. The Proofreading Taq Roche Expand High Fidelity^{PLUS} was used to try to reduce recombination events during PCR but it produced blunt ended sequences that required tailing and overall did not seem to improve on the performance of Invitrogen Platinum Taq.

Formation of mosaics by cloning heteroduplexes (Longeri et al 2002) is an important problem due to the high frequency of MHC heterozygotes (Jansen and Ledley 1990). It

is exacerbated by using primers that are probably not specific to one locus. Heteroduplexes are formed when two single strands of DNA from highly similar but distinct alleles re-anneal together in the final PCR cycle. When cloned the repair mechanisms in the *E. coli* for vectors are unable to differentiate a heteroduplex from a homoduplex. This leads to the situation where a heteroduplex plasmid is "repaired" and results in the formation of a product combining two different DNA sequences. This resulting new sequence is them amplified by the *E coli,* and can easily though incorrectly be assumed to be a "new" allele. In a wild population where a comparison to the alleles of the parents is unlikely to be available, detecting these artefacts requires a systematic approach. The SURE®2 Supercompetent cells which have the repair mechanisms disabled should reduce this problem occurring.

The primers were designed to flank exon 2 of the Class II B PBR instead of using primers designed for the conserved motifs internal to exon 2 for two reasons: 1) to minimise missing functionally important polymorphisms, and 2) to decrease the chances of amplifying more than one locus. In mammalian MHC with a more divergent evolution, many Class II B PBR primers are effectively designed by this approach. However avian MHC seems to have a more convergent pattern of evolution (Edwards et al 1998) and more than one locus may tend to be amplified by the primers (Zoorob et al 1993). As most kiwis tested (27/36) were found to have more than 2 sequences it is possible that more than one loci was sequenced, which is not an uncommon problem in birds (Edwards et al 1995b; Miller and Lambert 2004a, 2004b). It is hoped that future work sequencing more if not all the Kiwi MHC genome will identify the exact class I and II organisation and how many loci in each haplotype. Placing the primers further from the intron/exon boundary may have avoided this but it could also reduce the chances of detection of rare alleles in the desired locus (Edwards et al 1995b). Some researchers consider that the potential diversity of artefacts sequences in a clone library increases both with the number of sequence variants present in the original PCR and with the number of variable nucleotides in the sequences (Thompson et al 2002). This would indicate if the primers in a species are working at more than one loci there is an even greater amount of artefacts generated, so this project was careful to set high

criteria before accepting a sequence. The number of DRB loci can vary between and within species' haplotypes, it can only be said these results are likely MHC Class II DRB alleles and not to attribute specific loci for each allele.

The Nomenclature applied to the DNA sequences is derived from ISAG/IUIS-VIC Comparative MHC Nomenclature Committee (Ellis et al 2006), with the LSK (*Apteryx owenii*) prefixed by Apow, NIB (*Apteryx mantelli*) becoming Apma and Rowi (*Apteryx rowi*) becoming Apro. Due to the occurrence of artefacts during processing the criteria applied required a sequence (Class II exon 2) to either occur three times in two separate PCR's from a single bird or it is found in two birds, before it is recognised as part of an allele. This is based on the criteria used in other species like the DLA in dogs (Kennedy et al 2000). Some sequences were only found in clones from a single PCR, these sequences fail to meet the criteria for allele identification and are likely to represent artefacts of the PCR or cloning, however they may be rare alleles which are more likely to be missed by these criteria. The same criteria of sequence recognition as an allele is used applied to pseudogene recognition.

Pseudogenes are commonly found in mammalian MHC. They appear to be less common in avian MHC and this has been attributed to its more minimal nature (Kaufman et al 1999). Pseudogenes have been attributed to gene conversion and the "life and death" cycle of genes (Nei et al 1997). It is difficult to rule out any of the kiwi DNA sequenced here from potentially being part of a pseudogene. MHC pseudogenes have been described that are non-functional because of mutations in the controlling regions outside the gene rather than because of deleterious mutations within the exons (Zoorob et al 1990). Several suspected MHC pseudogene sequences were found in more than one animal e.g., two sequences were found in NIB that had a stop codon in the middle of the exon 2 sequence. Other kiwi MHC pseudogenes were identified where the PBR coding region was cut short and an unrelated sequence commenced.

Chapter 5

Polymorphism of the Major Histocompatibility Complex in the Kiwi (*Apteryx spp.*)

"In its birds each generation has but a life time interest; no more than sea and sky do they belong to any period. They are property entailed and to be transmitted age to age inviolate" (Guthrie-Smith 1914)

Introduction

The MHC is pivotal to the ability of the immune system of vertebrates in differentiating self from non self, and has been called "the centre of the immune universe" (Trowsdale 1995). MHC genes are the most polymorphic in the human genome. The extreme levels of polymorphism in classical MHC genes (Hedrick 1994; Hughes and Hughes 1995) are non-random in distribution and concentrate in the "Protein Binding Region" (PBR) of Class I & II (Hughes and Nei 1988). The study of the fitness effects of pathogens and the costs of the adaptive immune response has motivated interest in MHC and its polymorphism with emphasis on ecology and evolutionary biology rather than immunology (Hess and Edwards 2002). O'Brien (1985) was the first to suggest the possibility that a lack of MHC polymorphism may reduce a population's viability, and that reduced MHC diversity may leave an endangered population or species susceptible to epidemic diseases. Research has been performed on the amount of MHC polymorphism at the protein binding region (PBR) in a range of species, many of which are endangered. This thesis examines the characteristics of MHC polymorphism with respect to conservation in three kiwi species.

This project has developed the molecular tools for the characterization of MHC class II B genes in *Apteryx spp.* The second exon of class II B genes encode for the functionally important PBR which contain a number of highly polymorphic subdomains that diversify by point mutation, and gene conversion (interallelic and interlocus) (Gyllensten et al 1991; She et al 1991). Although an iconic bird in New Zealand all kiwi

species (*Apteryx spp.*) are threatened, and have undergone population decline since the arrival of humans. An understanding of kiwi MHC polymorphism may help in conservation management decisions relating to translocation, biosecurity and breeding strategies. Westerdahl (2000) suggested migratory birds would have higher levels of MHC polymorphism than non-migratory birds. This raises questions about whether the non-migratory and flightless kiwi would tend to low levels of MHC polymorphism and if so is it susceptible to emerging infectious diseases (EID)? We were unable to find details published MHC data on any *Apteryx spp.* or other Paleognathae MHC to date. Comparisons between Neognathae MHC, kiwi Paleognathae MHC and mammalian MHC may elucidate more about the evolutionary trends in MHC. Kiwi MHC may be especially interesting as the kiwi (*Apteryx spp.*) has shown several mammalian tendencies for a bird (Sale 2005).

MHC Polymorphism and Conservation of Endangered species.

The importance of MHC polymorphism to disease response at an individual and population level has been discussed in Chapter One. Some consider that because of its role in the immune system the maintenance of MHC polymorphism should be important to conservation management of declining and endangered populations (O'Brien and Evermann 1988; Hughes 1991). There are many threats to our endangered species such as habitat alteration and destruction, pollution, resource exploitation, and introduced predators and competitors. More recently the importance of existing and emerging exotic diseases has come to the fore (Deem et al 2001; Lafferty and Gerber 2002), with many examples including the loss of 5,000 lowland gorilla (*Gorilla gorilla gorilla*) to Ebola virus (Bermejo et al 2006). The H5N1 avian influenza pandemic has not only killed many birds including bar headed geese (*Anser indicus*) but it also killed tigers (*Panthera tigris*), leopards (*Panthera pardus*) (Keawcharoen et al 2004), and Owston's civet (*Chrotogale owstoni*) (Roberton et al 2006). The exact magnitude of the threat of a given pathogen to a specific MHC depauperate population needs to be assessed on a case by case basis. Assessing a declining or an endangered population's MHC polymorphism level may indicate if generally the population is at greater or lesser risk to an infectious disease, and it can be a contributing factor in breeding programmes.

Moreover initiating a breeding programme for endangered species to maintain their MHC diversity when they are in the midst of an epidemic is too late; to be effective it must occur before the problem.

O'Brien (1985) was the first to suggest the possibility that a lack of MHC polymorphism may reduce a population's viability, and that reduced MHC diversity may leave an endangered population or species susceptible to epidemic diseases. Hughes (1991) went a step further in emphasizing the importance of maintaining MHC polymorphism in endangered species by proposing that a main goal of captive breeding programmes should be the maintenance of the allelic diversity at loci like MHC, under balancing selection. A consequence of this emphasis could be increased loss of heterozygosity at other loci, but Hughes (1991) considered that as the "vast majority of genetic polymorphisms are selectively neutral" that such a loss is of little concern compared to the loss of a MHC diversity in a vertebrate. This recommendation caused some disagreement, especially as it could result in the inadvertent fixation of a deleterious non-MHC allele (Gilpin and Wills 1991; Vrijenhoek and Leberg 1991). Although the importance of MHC diversity was not challenged, another concern was the difficulty in determining which MHC alleles may be advantageous, neutral or even detrimental (many HLA alleles are associated with autoimmune diseases) (Miller and Hedrick 1991). However, it may be possible to make an educated guess as to the importance of particular MHC alleles given the selection model of Ohta (Ohta 1991) in which heterozygotes that differ greatly in amino-acid sequence have higher fitness, and thus one could favour these allele combinations as a modification of Hughes' proposal.

Currently accepted breeding management approaches for captive populations such as minimizing mean kinship emphasize avoiding inbreeding to maximise overall population genetic diversity, and this approach should also retain MHC diversity (Hedrick 2003). The importance of genetic diversity over the long term is the maintenance of adaptive evolutionary potential, and over the short term maintenance of reproductive fitness making it a primary focus for conservation genetics (Frankham et al 2002). However, it is possible to pair individuals with a rare allele (like an MHC allele)

to mates selected to minimize mean kinship (Miller 1995). This combined emphasis although not as effective as breeding to a single goal, reduces the overall loss of heterozygosity that can occur if a breeding strategy is aimed at just increasing the frequency of rare MHC alleles.

It is difficult to predict how a given population will respond to inbreeding pressures, especially under variable environmental conditions (Hedrick and Kalinowski 2000). A depression of the immune response, as measured by a reduced cell mediated immunity response to phytohaemagglutinin challenges, can be a side effect of inbreeding (Reid et al 2003; Hale and Briskie 2007). Generally inbreeding depression effects increase under stressful conditions (Armbruster and Reed 2005) compounding the problem as a species in decline are often under multiple stresses. Spielman et al. (2004) showed (in *Drosophila melanogaster*) with a meta-analysis approach that inbreeding and loss of genetic diversity decreased disease resistance and this was due to specific polymorphic loci affecting disease resistance, rather than a consequence of generalised inbreeding depression. Inbreeding depression effects are expected to be greater for wild populations than captive populations where most inbreeding research is conducted, as they are exposed to many different pathogens and inbred populations that are relatively resistant to one pathogen are likely to be susceptible to other unrelated pathogens (Frankham et al 2002; Spielman et al 2004).

Several populations with low levels of MHC polymorphism have been rebounding and are currently increasing in size e.g., Chatham Is Black Robin (*Petroica traversi*) (Miller and Lambert 2004b), Swedish Fallow deer (*Dama dama*) and Musk ox (*Ovibos moschatus*) (Mikko et al 1999), Northern Elephant seal (*Mirounga angustirostris*)(Weber et al 2004) and Eurasian beaver (*Castor fiber*) (Balik et al 2005). The Northern Elephant Seal has been proposed as a species that has overcome inbreeding depression and adapted to low genetic variability. The apparent success of these increasing populations could be due to selection on MHC loci only being weak (Klein et al. 1993) or that the selection force is dynamic with temporal and spatial variations. So are these populations just "an accident waiting to happen" i.e., they could

be dangerously exposed when challenged by a new pathogen. Jarvi has shown that the native Hawaiian honeycreepers (*Drepanidinae*) have low levels of Class II B polymorphism and are facing a major extinction threat from multiple factors including the recent introduction of avian malaria (Jarvi et al 2004). It may not be possible to identify one pathogen as the single contributing factor in an endangered species decline, however a shared pathogen introduced from another more common competing species, particularly if the endangered species has low resistance, could well be a fatal combination resulting in extinction (Arkush et al 2002).

MHC monomorphism does not preclude life, reproduction or population growth, as can be seen in rebounding populations like the Chatham Is Black Robin (*Petroica traversi*). What MHC monomorphism may do is make an entire population potentially vulnerable to pathogens that avoid or subvert the immune response based on the common MHC type (Parham 1999). The benefit of MHC polymorphism may be in reducing the risk that episodes of disease will develop into catastrophic epidemics. This has lead to researchers making recommendations on specific endangered species management based on their MHC. Along with low levels of MHC diversity (class II B DRB) the South African bontebok (*Damaliscus pygargus pygargus*), also contains some alleles not present in other subspecies (van der Walt et al 2001). This population has in the past been susceptible to parasites leading Van der Walt et al. (2001) to recommend that these potentially advantageous alleles should be actively retained by interbreeding with other *Damaliscus pygargus* subspecies. Genetic restoration has been accomplished in the inbreed Florida panther (*Puma concolor coryi*) by introducing Texas cougars (*Puma concolor*) (Hedrick 2005). The Crested ibis (*Nipponia nippon*) also has low MHC diversity (class II B) and their susceptibility to disease lead Zhang et al (2004) to recommend preserving their remaining diversity to be part of any management and translocation decisions. But there is much to be established before it will be possible to predict the effects of introducing migrants for genetic restoration to a population (Ingvarsson 2001; Tallmon et al 2004). Without more specific information on a particular species with a specific disease epidemiology it is difficult to make more than such general recommendations on maintaining MHC diversity.

MHC polymorphism can get so low in a population that skin grafts between individuals are not rejected, as seen in cheetahs (*Acinonyx jubatus*) (O'Brien et al 1985) and pocket gophers (*Thomomys bottae*) (Sanjayan et al 1996). An example of how dangerous this low a level of MHC polymorphism can be, may be occurring in the Tasmanian Devil *(Sarcophilus harrisii).* The Tasmanian Devil population appears to have limited MHC diversity and may be driven to extinction (unless a successful intervention occurs) by an emerging disease, a transmissible facial tumour (McGlashan et al 2006; Siddle et al 2007). And it is worth remembering although transmissible tumours are very rare, infectious pathogens are not.

The Major Histocompatibility Complex (MHC) of Kiwi

At the start of this project (and currently) we have found no published data on the MHC in Kiwi or published data on MHC in Ratites or Tinamou has been found. The DNA sequence of kiwi MHC will enable comparisons of Paleognathae MHC to Neognathae and mammalian MHC and may elucidate more on the evolution of MHC.

Although recognised as taonga (a treasure) in New Zealand, there are many gaps in our knowledge of the kiwi and its diseases (reviewed in Chapter 2). The conservation status of all five recognised kiwi species is of concern, with an article in Time magazine wondering if they were "On Their Last Legs?"(Robinson 1998). Knowledge of an animals' MHC haplotype can also be used in the management of translocation security and breeding programme decisions. An analysis of the amount of MHC polymorphism in various kiwi populations can be used to identify whether any of the fragmented populations are depauperate and therefore potentially at greater risk than other populations. Populations with novel alleles can be identified and used to inject new alleles into those with limited diversity. The ability to identify a kiwi's haplotype could potentially be used in surveillance of wild populations. An otherwise occult epidemic may be detected by an ongoing change in the frequency pattern of MHC alleles indicating a disease process is reducing the population rather than something like predators. Or possibly examining the expression levels of DRB MHC in a population to

monitor current immune activity in a population (Bowen et al 2006). If there is a link between MHC and mate choice in kiwi, using it to choose partners may increase the success of breeding programmes.

Methods and Materials

1.0 Sample populations.

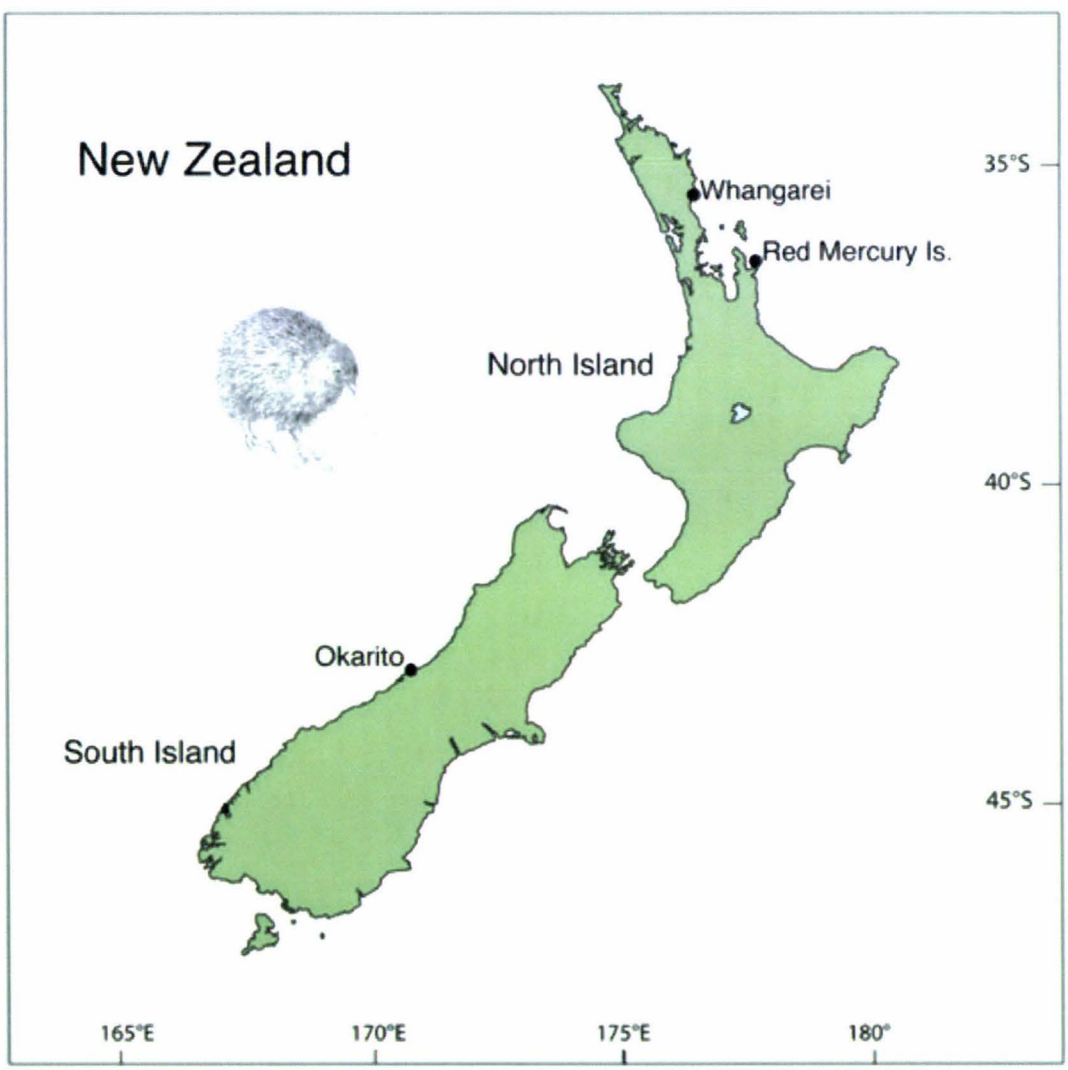
Kiwi feather samples were taken from three different kiwi populations shown in Figure 5.1.

1.1 The Little Spotted Kiwi (LSK) (*Apteryx owenii*). The LSK feather samples were collected from Red Mercury Is (36°37'S, 175°55' E). DNA was extracted for analysis from eight birds. The main population of LSK is on Kapiti Is (40°51' S, 174°55' E) and is estimated at about 1200 birds. There is a disagreement on the origin of the Kapiti Is birds whether they were originally on the island or were introduced by humans (especially in the early 1900's) (Jolly and Colbourne). The other long standing population was on D'Urville Is (40°50' S, 173° 50'E). It dwindled to near extinction and was interbred with several Kapiti Is birds in the 1980's. The D'Urville Is birds have since been transplanted to Long Island (41°08'S, 174°15'E) and had in 2002 had an estimated minimum population of 20 birds (Colbourne 2005). In the last 20 years several other offshore islands have had LSK populations established. In June 1983 six male and six female LSK were transported from Kapiti Is to start the Red Mercury Is population (Jolly and Colbourne 1991). A 1996 survey revealed it had grown to a minimum size of 30 individuals (Colbourne and Robertson 1997). Tiritiri Matangi (36°36'S, 174°57'E) had a total of 16 LSK introduced from Kapiti Is in the 1990's, and by 2002 the population was estimated at 50 birds (Colbourne 2005). Hen Is (35°58'S, 174°43'E) had a total of 38 LSK transferred from Kapiti Is between 1988 & 1989. In 1995 the population was estimated as 50 birds (Colbourne and Robertson 1997). Twenty LSK were placed in the predator free Karori Sanctuary on the North Island in June 2000, there is now an estimated 50-70 birds. Although the LSK has reversed its rapid decline in numbers, these results indicate careful examination and management of its genetic diversity are needed.

1.2 The Okarito Rowi *(Apteryx rowi).* The Rowi feather samples were gathered from Okarito in the South Is, which has a population of ~250 birds. The Okarito Kiwi Sanctuary covers 11,000-hectares of forest near Okarito on the West Coast of the South Island in Westland National Park. DNA of suitable quality was extracted from 18 different birds' feathers.

1.3 The North Island Brown Kiwi (NIB) (*Apteryx mantelli*) . The NIB feather samples came from a population near Whangarei (Fig 5.1). The DNA extracted from ten birds was suitable for analysis. The complete distribution of the estimated 25,000 NIB is shown in Figure 2.1 and Table 2.1.

Figure 5.1 The Location of the Three Kiwi populations sampled.



The North Island Brown Kiwi feather samples came from a population just to the north of Whangarei. Red Mercury Island was the source of Little Spotted Kiwi feather samples. Rowi feather samples were taken from Okarito in South Island.

2.0 Method.

DNA was extracted from the feathers as described in Chapter 3 section 3.1.1.
The DNA was then either stored overnight at 4°C or a PCR was performed that day.
The Class II B exon 2 primers, KiwintA1F & KiwintA2R were used as described in chapter 3 section 3.3.5 on samples from the three wild Kiwi populations.

The PCR product was ligated with Invitrogen pCR 2.1® Vector (see chapter 3 section 3.6.4) and transformed into Stratagene SURE®2 Supercompetent cells (see chapter 3 section 3.6.5). Suitable colonies were checked for inserts and fresh colonies and the resulting plasmids were amplified with TempliPhi™ (see chapter 3 section 10.0) and then sequenced (see chapter 3 section 3.12.2) with Big Dye Termination mix 3.1 (BDT 3.1) and the M13 Reverse primer on the ABI 3730 Genetic Analyzer.

The process developed to sequence the Kiwi Class II B exon 2 (DRB-like) region was also developed to minimise unwanted recombination events and maximise the quality of the final sequences. The process involved a PCR of the required DNA, cloning with SURE®2 Supercompetent cells, amplifying the resulting plasmids with TempliPhi™ and then sequencing. Using SURE®2 cloning and TempliPhi™ minimised the generation of error (artefacts) especially from unwanted recombination events during processing and produced sequences with high quality base calling scores. Stratagene SURE®2 Supercompetent Cells are designed to stop unwanted DNA rearrangement events by lacking components of pathways that catalyse the rearrangement of nonstandard structures in DNA. This inability to repair such events during DNA plasmid amplification in these cells should reduce recombination artefacts in the MHC DNA finally sequenced. The TempliPhi™ rolling amplification of circular DNA at a constant temperature with Φ29 DNA polymerase may reduce recombination events and heteroduplex formation (Dean et al 2001; Nelson et al 2002). The stock solutions for these PCR reactions were prepared in the ancient DNA lab using Gibco water. This

laboratory is physically separate from the main Allan Wilson Centre laboratory on Albany campus of Massey University, so as to avoid any contamination by PCR products produced previously in this project. The quality of the sequences was analysed by base calling in Sequencher 4.6 based on PHRED scoring (Ewing and Green 1998; Ewing et al 1998).

In total the DNA from 18 Rowi, ten NIB and eight LSK individuals were used. Each individual's DNA was amplified by two separate PCRs. Each PCR was then cloned and five colonies with suitable sized inserts from each PCR, i.e., 10 from each animal, were sequenced. The criteria for identifying and naming alleles and pseudogenes are discussed in chapter 4.

Results.

Alleles & Pseudogenes

Several putative alleles and pseudogenes were found in Kiwi. Although the sequenced area is exon 2 and not the entire length of Class II B, for ease of reference and to be consistent with other similar investigations, it will be referred to as an "allele" (Kennedy et al 2000). The numbers found and the size in each species is listed in Table 5.1. Several sequences have been identified as suspected pseudogenes in NIB & rowi, due to either stop codons in the middle of the sequence or they contained large anomalous areas of sequence.

Table 5.1 The number and sizes of the putative alleles sequenced in Kiwi.

Species	No birds	No of alleles	Size 281 bp	Size 284 bp	Suspected pseudogenes
LSK alleles	8	4	3	1	0
NIB alleles	10	11	6	5	2
Rowi alleles	18	14	8	6	3

There is size variation in the exon 2 segment of the sequences, i.e., the sequence amplified with the introns either end removed. The 284 bp sequences have an exon 2 that is 267 bp long. The 281 bp sequences mainly have a 264 bp long exon 2, but a sequence in the LSK is 258 bp long. The sequences for LSK are in Appendix B, NIB in Appendix C and rowi sequences are in Appendix D.

Putative Loci

The sequences have a shared homology with Class II DRB 1-like sequences, based on comparison with similar sequences found in the NCBI database. The results show most birds (27/36) had more than two "alleles" indicating more than one locus has been amplified. The number of DRB-like loci can vary between and within species' haplotypes (heterogeneity in gene number) e.g., DRB region in primates (Brandle et al 1992) and class II MHC in cichlid fish (Malaga-Trillo et al 1998).

An attempt was made to estimate the number of loci in each species by generating a neighbour joining tree for each species based on the full sequences identified as alleles (Tamura et al 2007). Figure 5.2 shows possible LSK loci, Figure 5.3 is the NIB and Figure5.4 shows the putative loci results for rowi. The Neighbour-Joining (NJ) method of tree building infers evolutionary history, and shows branch lengths proportional to evolutionary distances with all gaps eliminated from the dataset (Saitou and Nei 1987; Tamura et al 2004). The individual birds of each species were numbered, and the alleles found in each bird were correlated to the Neighbour Joining tree. A series of lines were then drawn allocating loci based on several guidelines. Theses were to find the earliest divisions in the tree where a single bird can not contain more than two alleles at a given loci and creating the minimum number of divisions (loci). The guidelines assume that the sequences are paralogs and the loci formed by gene duplication events.

The results showed a good correlation of the NIB to three loci each (Fig5.3). The results showed two probably three loci for LSK (Fig 5.2). The relationship of the rowi to loci (Fig 5.4) on this basis was not as clear with the analysis implying 5 (maybe 6 loci) and the suspected divisions not always correlating well with the earliest branching points. The more loci present the smaller the amount of polymorphism shown at each site, and the larger the sample size needed to recover a reliable tree topology.

Interestingly the NJ tree for rowi aligns with the earliest division being between the 284 and the 281 bp sized sequences. Putative loci, R1 264 bp, R2 264 bp, R3 264 bp, R4

267 bp, & R5 267 bp. The LSK also shows its earliest division was between 284 and 281 bp, and the last division was within the 281 bp group producing the two sizes of exon 2 (264 and 258 bp). Putative loci, L1 258/264 bp, L2 264 bp & L3 267 bp However the NIB allele tree does not show its earliest division as being between 281 and 284 bp sequences. The two different sequence sizes are spread out among all suspected loci for NIB. Putative loci, N1 264/267 bp, N2 267 bp & N3 264/267 bp. In Figure 5.5, an NJ tree of all kiwi sequences, the earliest split is between the 284/267 bp & 281/264 bp groups, except for one allele Apma 008. Figure 5.5 does not neatly relate a given loci of one species to another species loci. However using MEGA 4.0.2 (Tamura et al 2007) to align the sequences it looks like a single indel of three contiguous base pairs (TCA at positions 237, 238 & 239) differentiates the 281/264 bp and 284/267 bp groups. As shown in Fig 5.5 this event appears to have occurred prior to speciation, so the two groups are orthologous. In contrast the deletion event that created Apow 001 appears to have removed a group of six contiguous base pairs at the start of exon 2. This is the only 281/258 bp sequence found in kiwi and possibly is a post-speciation event in the LSK.

Figure 5.2 Putative loci for Class II MHC DRB-like alleles in Little Spotted Kiwi (*Apteryx owenii*).

Phylogenetic tree of four alleles and their distribution in eight little spotted kiwi. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987) . The optimal tree with the sum of branch length = 0.384 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 281 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al 2007).

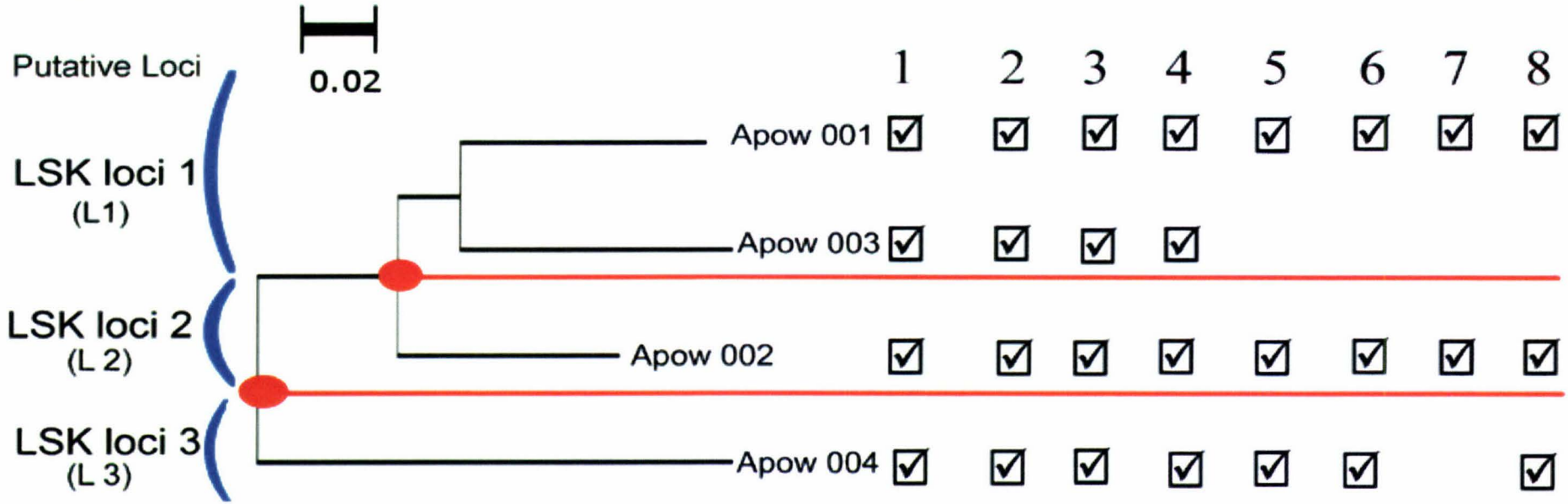


Figure 5.3 Putative loci for Class II MHC DRB-like alleles in North Island Brown Kiwi (*Apteryx mantelli*).

Phylogenetic tree of 11 alleles and their distribution in ten North Island brown kiwi. For the NIB an evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987). An optimal tree with the sum of branch length = 0.741 is shown. The analysis of data is the same as Fig 5.2.

Figure 5.4 Putative loci for Class II MHC DRB-like alleles in Rowi.

Phylogenetic tree of 14 alleles and their distribution in 18 rowi (*Apteryx rowi*). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.790 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis of data is the same as Fig 5.2.

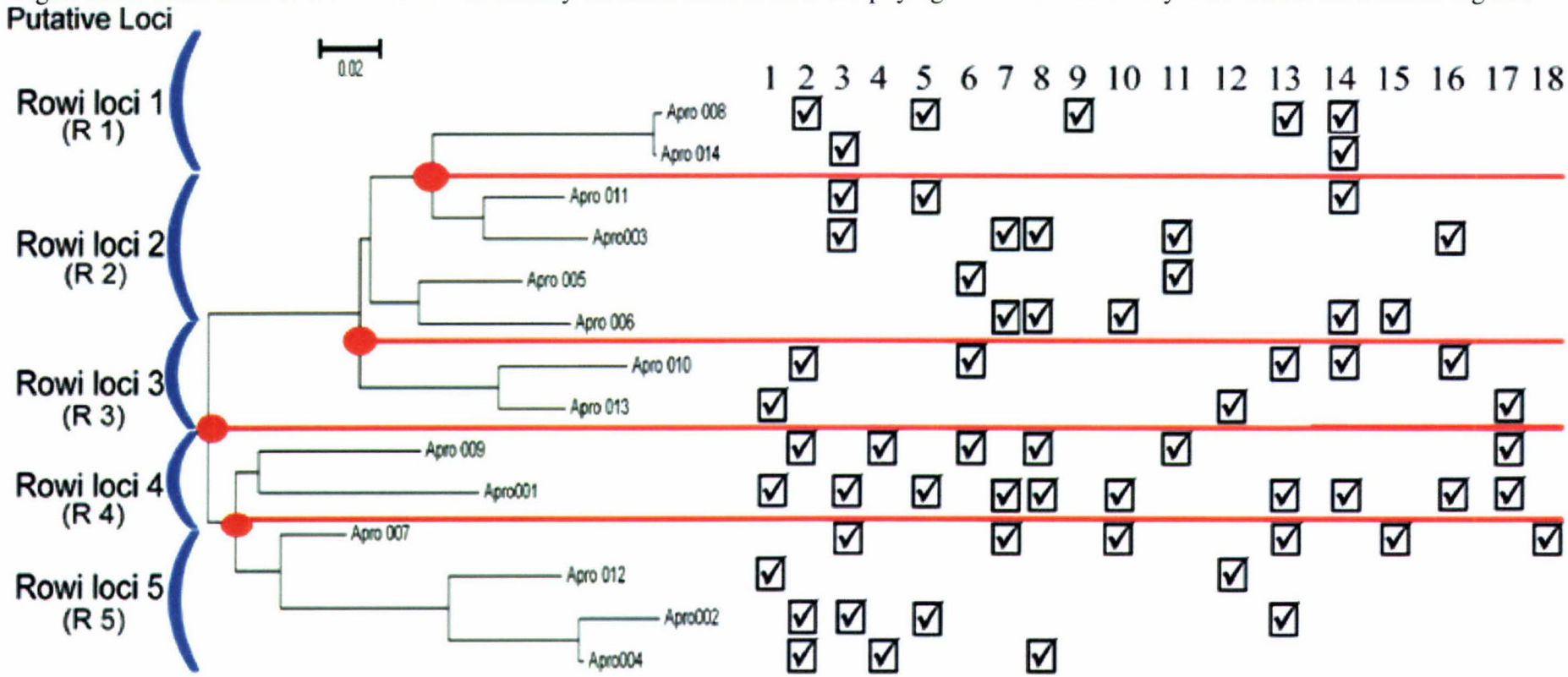
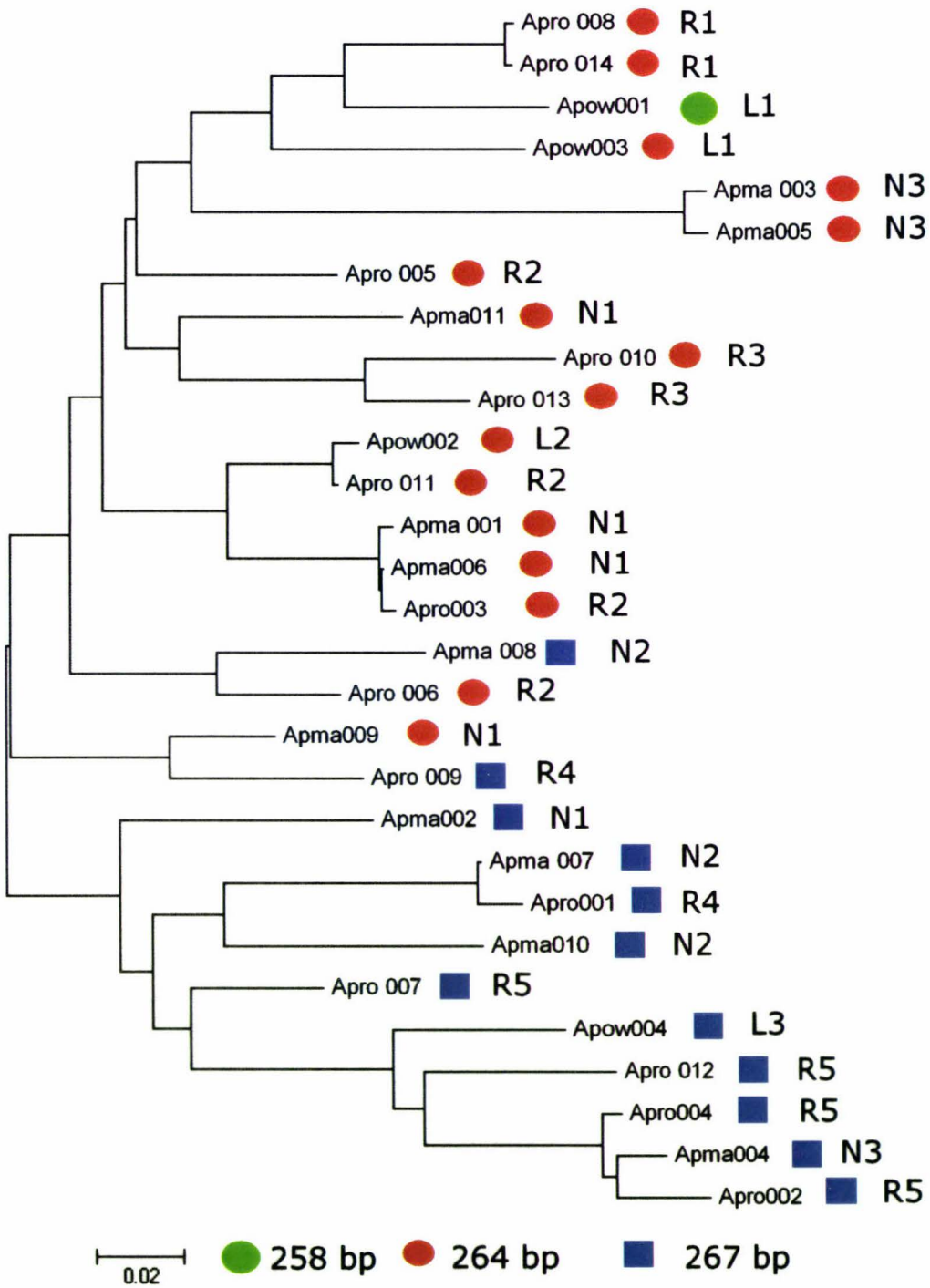


Figure 5.5 A Neighbour joining tree of the Kiwi alleles, showing exon 2 sequence sizes and putative loci.



The evolutionary history was inferred using the Neighbor-Joining method(Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.35583318 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary

distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969)and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 281 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al 2007).

The size of the exon 2 sequence is shown as a green circle for 258 bp, a red circle for 264 bp and a blue square for 267 bp. The putative loci are recorded as L1, L2 & L3 for LSK loci: N1, N2 or N3 for NIB and R1,R2 ,R3, R4, & R5 for rowi. They relate to the loci shown in Figures 5.2 – 5.4.

The size and frequency of the alleles.

Table 5.2 shows that pattern of frequency of the alleles in this sample is intermediate with no clearly dominant sequence except in the LSK. The LSK show very high frequency of all alleles in the sample, possibly due to lack of MHC polymorphism at any loci. The suspected pseudogenes are also show an intermediate frequency (11-30%) pattern.

The entire DNA sequences for each given allele and the section identified as exon 2 of each species is presented in the Appendixes. The LSK sequences are in Appendix B. The NIB sequences are in Appendix C and the Rowi are in Appendix D. The exon 2 from the full 284 bp sequences all had 2 open reading frames (ORF), but only the one ORF that correlates to Class II MHC sequences and this one was analysed.

Table 5.2 The size and frequency of the alleles in each species of Kiwi.

Alleles by species	Allele ID	size of sequence (bp)	size of exon 2	% Frequency of allele in sample
LSK	Apow 001	281	258	(8/8) 100.0
	Apow 002	281	264	(8/8) 100.0
	Apow 003	281	264	(4/8) 50.0
	Apow 004	284	267	(7/8) 87.5
NIB	Apma 001	281	264	(2/10) 20.0
	Apma 003	281	264	(4/10) 40.0
	Apma 005	281	264	(3/10) 30.0
	Apma 006	281	264	(4/10) 40.0
	Apma 009	281	264	(2/10) 20.0
	Apma 011	281	264	(2/10) 20.0
	Apma 002	284	267	(3/10) 30.0
	Apma 004	284	267	(2/10) 20.0
	Apma 007	284	267	(2/10) 20.0
	Apma 008	284	267	(2/10) 20.0
	Apma 010	284	267	(2/10) 20.0
NIB pseudogenes	Ap nib DRB8	281	129	(3/10) 30.0
	Ap nib DRB2	281	186	(3/10) 30.0
Rowi	Apro 003	281	264	(5/18) 27.8
	Apro 005	281	264	(2/18) 11.1
	Apro 006	281	264	(5/18) 27.8
	Apro 008	281	264	(6/18) 33.3
	Apro 010	281	264	(5/18) 27.8
	Apro 011	281	264	(3/18) 16.7
	Apro 013	281	264	(3/18) 16.7
	Apro 014	281	264	(1/18) 11.1
	Apro 001	284	267	(10/18) 55.6
	Apro 002	284	267	(2/18) 22.2
	Apro 004	284	267	(3/18) 16.7
	Apro 007	284	267	(6/18) 33.3
	Apro 009	284	267	(6/18) 33.3
	Apro 012	284	267	(1/18) 11.1
Rowi pseudogenes	Ap row DRB5	281	186	(5/18) 27.8
	Ap row DRB6	283	103	(1/18) 11.1
	Ap row DRB16	283	103	(5/18) 27.8

% Frequency in sample is the number of birds in the sample analysed for that species found to have the allele.

Gene conversion

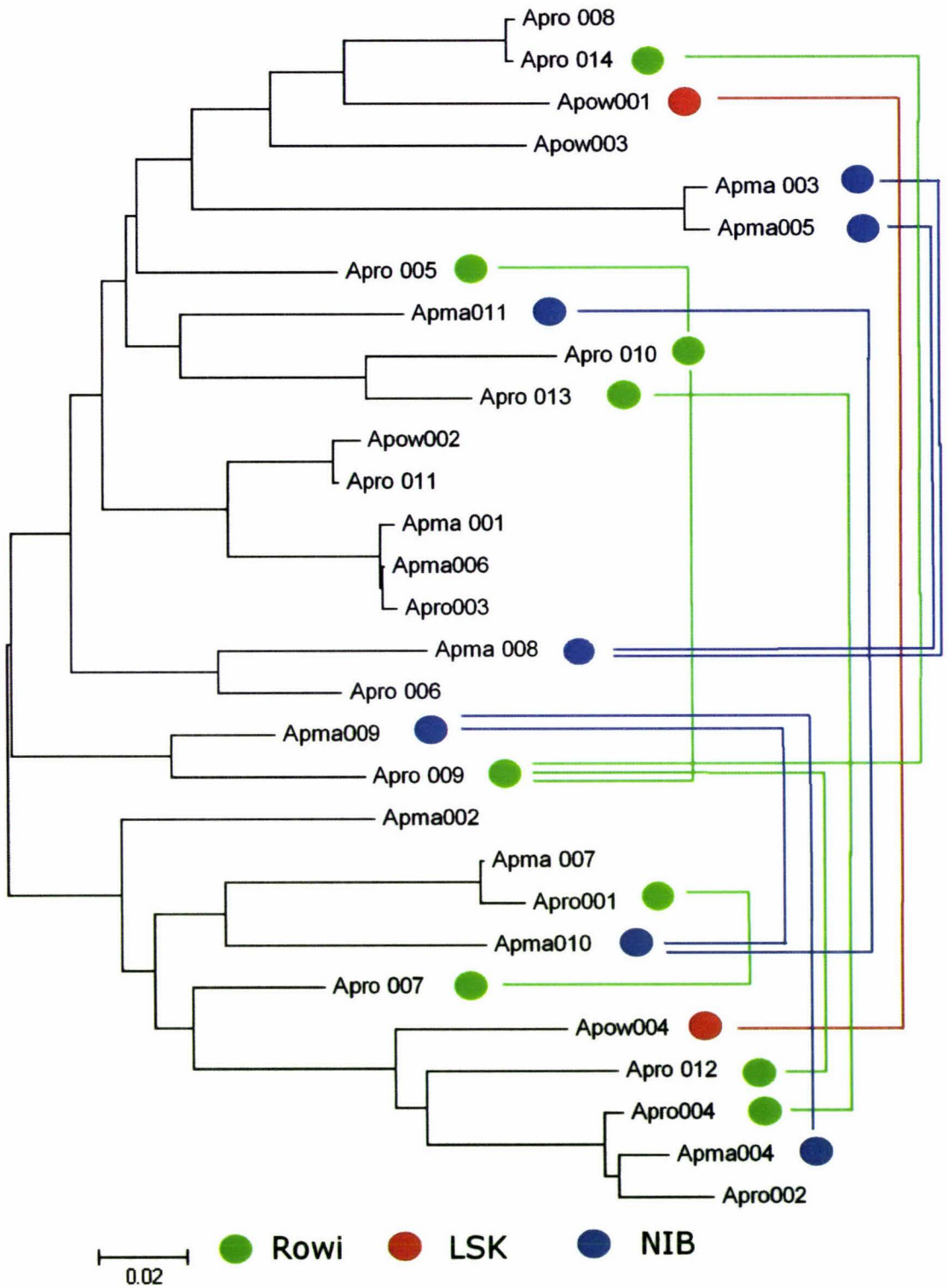
Gene conversion has often been suggested as a cause of some of the patterns observed in MHC sequences, but its relative importance is contested (reviewed (Martinsohn et al 1999)). In Table 5.3 the occurrence of gene conversion was estimated using the software GENECONV version 1.81a (Sawyer 1999). GENECONV analyses the distribution of nucleotide differences to detect gene conversion events by looking for stretches of nucleotides in a pair of sequences that are more similar to each other than would be expected by chance (Drouin 1999). In order to avoid the confounding effects of selection, the exon 2 region of each sequence for each species was analysed for gene conversion events using silent sites (those nucleotides that can change without changing the resulting amino acid) only. Using this restriction, and analysing each species separately, there was no evidence of any gene conversion events among the Kiwi MHC alleles. Allowing silent and non-silent sites to be included in the analysis of coding regions means that other forms of selection could influence the result. Searching all kiwi species across the exon 2 region identified some putative gene conversion events within each species when all sites were included. They were considered statistically significant with the simulated global P value, based on 10,000 permutations of the original data, less than 0.05. G scale values of 0, 1, and 2 were used, allowing for varying levels of mismatches (i.e. subsequent mutation) within the gene conversion event to be taken into account. A G scale value of 0 allows for no mismatches within the converted region, whereas values of 1 and 2 allow for progressively more mismatches i.e. more lax comparison allowing for more mutations after the gene conversion event.

Table 5.3 Predicted Gene Conversion Events between Kiwi in the MHC class II.

Sequence 1	Sequence 2	Sim P	Begin	End	Length	No. bp different	G scale
LSK							
Apow 001	Apow 004	0.0483	121	165	45	0	0
Apow 001	Apow 004	0.0000	71	165	95	3	1
Apow 001	Apow 004	0.0199	71	165	95	3	2
ROWI							
Apro 001	Apro 007	0.0414	192	258	67	0	0
Apro 001	Apro 007	0.0045	149	258	110	1	2
Apro 004	Apro 013	0.0171	127	176	50	0	0
Apro 004	Apro 013	0.0153	127	176	50	0	2
Apro 005	Apro 010	0.0062	202	267	66	0	0
Apro 005	Apro 010	0.0090	202	267	66	0	2
Apro 009	Apro 010	0.0204	150	235	86	3	2
Apro 009	Apro 012	0.0235	213	257	45	0	0
Apro 009	Apro 012	0.0309	213	257	45	0	2
Apro 009	Apro 014	0.0120	127	197	71	2	2
NIB							
Apma 003	Apma 008	0.0087	48	90	43	0	0
Apma 003	Apma 008	0.0069	48	90	43	0	2
Apma 004	Apma 009	0.0018	91	143	53	0	0
Apma 004	Apma 009	0.0000	91	176	86	1	1
Apma 004	Apma 009	0.0000	91	176	86	1	2
Apma 005	Apma 008	0.0062	48	90	43	0	0
Apma 005	Apma 008	0.0047	48	90	43	0	2
Apma 009	Apma 010	0.0073	128	188	61	0	0
Apma 009	Apma 010	0.0045	128	197	70	1	2
Apma 010	Apma 011	0.0006	150	199	50	0	0
Apma 010	Apma 011	0.0008	150	199	50	2	2

Sim P, simulated P values based on 10 000 permutations; Begin, the first nucleotide of the converted region; End, last nucleotide of the converted region, based on nucleotide numbering; Length, length of the converted region; No different bp, is the number of bp different in section of DNA translocated; gscale indicates the mismatch penalty.

Figure 5.6 Map of putative Gene Conversion events shown on Neighbour Joining tree of Kiwi sequences.



Each line links the sequences identified in Table 5.3 as being involved in a possible gene conversion event. Green circles and green lines represent rowi. Blue circles and lines represent NIB. Red circles and lines represent LSK. The Neighbour-Joining tree has the same parameters as those used for Fig 5.5. The gene conversion events appear to cross between the two sizes of exon II i.e., 264 bp and 267bp, which may explain why it is difficult to estimate the number of loci on a phylogenetic basis.

Network analysis

A NeighborNet Network analysis of the relationship of the alleles to each other (Figure 5.7 & 5.8) was produced using SplitsTrees4 (Huson and Bryant 2006). The NeighborNet split tree analysis was performed with uncorrected P distances, with ordinary least squares variance and the equal angle algorithm (Dress and Huson 2004.). The Neighbour-Net method (Bryant and Moulton 2002) is a hybrid of Neighbour-Joining and split decomposition and can visualise more complex phylogenetic relationships such as hybridization, horizontal gene transfer, recombination, or gene duplication and loss (Huson and Bryant 2006). In a split network, every edge is associated with a split of the alleles, but there may be a number of parallel edges associated with each split inferring that incompatible splits exist. A split decomposition network uses a distance matrix to produce weighted X-splits (Bandelt and Dress 1992). Multiple edges can be seen in relationships between sequences of DRB like Class II MHC within and between Kiwi (Fig 5.7) and between various bird families (Fig 5.8). Like the trees in Fig 5.5, Fig 5.7 shows no groupings based on the putative loci or species. This may be due to recombination events like gene conversion, multiple exchanges of DNA sections are known to occur in birds and fish obscuring assignment of MHC alleles to loci (Miller et al 2007). The pattern in kiwi of the MHC not grouping at species level is consistent with other species' MHC findings and may be due to transpecies evolution of polymorphism (reviewed (Klein et al 1998)).

Figure 5.7 A NeighborNet of the relationship between the putative alleles found in the three kiwi populations.

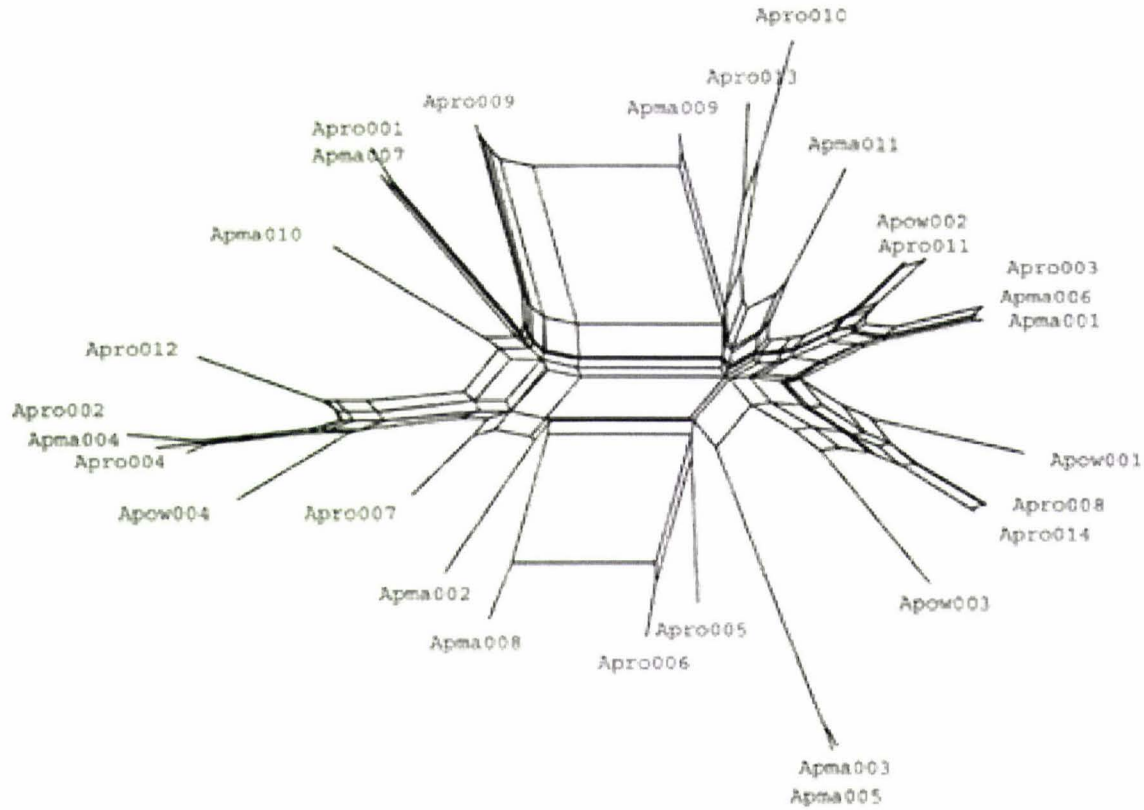


Figure 5.8 The NeighborNet analysis of Avian MHC Class II B exon 2.

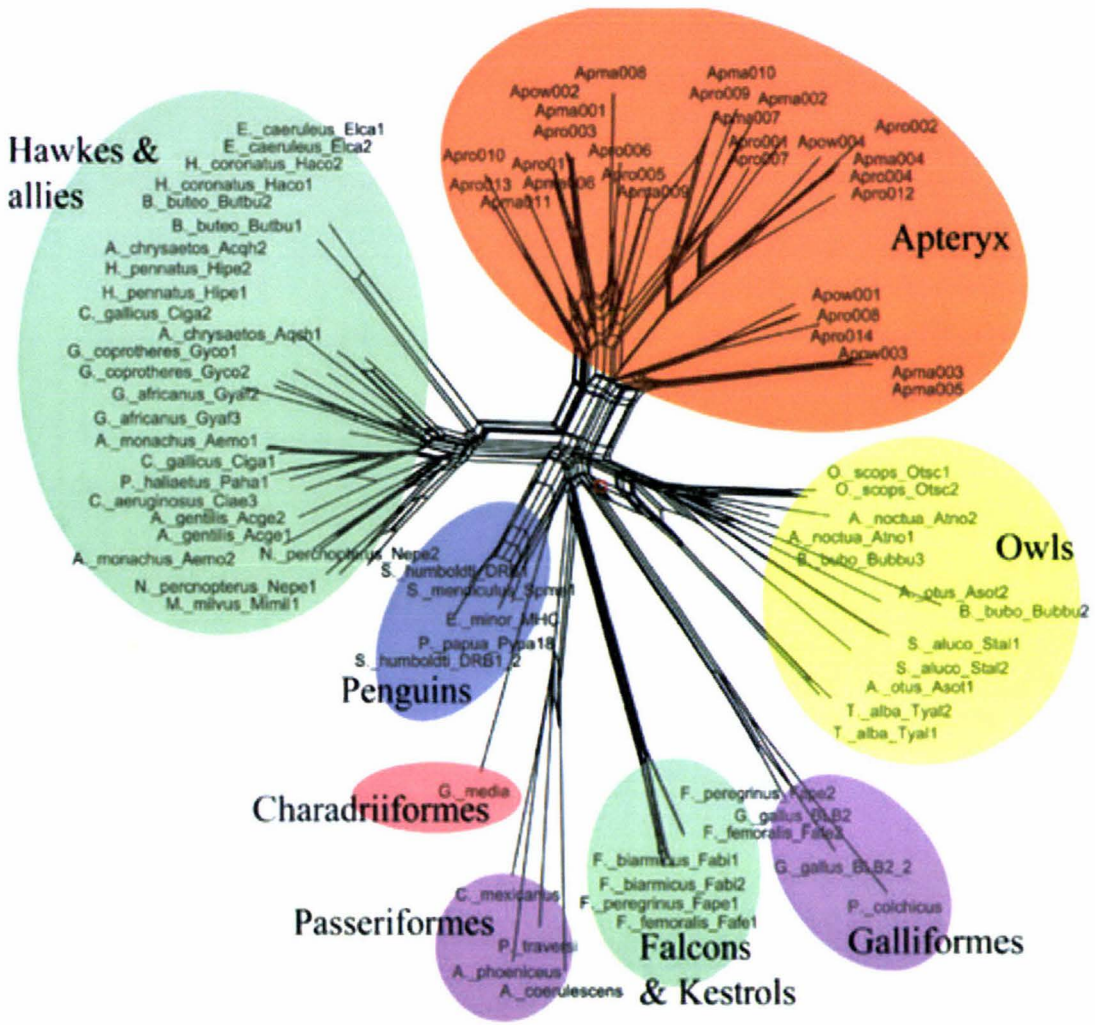


Figure 5.8 is a Neighbour-Net of MHC Class II B exon 2 from various avian families. It was generated using SplitsTree 4 (Huson and Bryant 2006). The sequences used are listed in Appendix G Table G.1. The Neighbour Net split tree analysis was performed with uncorrected P distances, with ordinary least squares variance and the equal angle algorithm (Dress and Huson 2004). The Neighbour-Net method is a hybrid of Neighbour-Joining and split decomposition, and is applicable to data sets with hundreds of taxa (Bryant and Moulton 2002). Neighbour-Net generates a weighted circular split system rather than a hierarchy or a tree, which can be represented in a splitsgraph. The bipartitions or splits represent incompatible or ambiguous signals in the dataset. The nodes generated do not necessarily represent ancestral species. When evolution is

assumed to be a tree like process linking a species to its closest ancestor evolutionary mechanisms such as hybridization, gene loss and duplication, horizontal gene transfer and gene conversion are not taken into account (Makarenkov et al 2006). Gene loss, duplication and recombination have been widely described in MHC (Nei et al 1997; Hess and Edwards 2002; Miller and Lambert 2004a) and this may explain the multiple parallel lines in Fig 5.8 & 5.7.

The network in Figure 5.8 shows the birds grouping together at the Taxonomy level of major Family and Family level in general. This is the same as the finding of Alcaide et al (Alcaide et al 2007). However the grouping of the avian relationships at species level is not consistent. The wide spread seen between the kiwi alleles may be due to more than one loci being present and a larger dataset for kiwi used or transpecies polymorphism and gene conversion.

Transpecies polymorphism

Transpecies polymorphism has been known to occur in the MHC, including in birds but more commonly in mammals (Jarvi et al 2004). Although the sequence for Apro 005 in Rowi was recorded in one sequence from a NIB it was not repeated in another PCR, so it cannot be confirmed in kiwi yet. However a mixture of transpecies polymorphism and gene conversion may explain the pattern in Fig 5.7 & 5.8. Where despite being only differentiated at the genus level Apteryx spp. show as wide a spread in the network analysis as other birds that are differentiated at the Family level e.g., Hawkes & allies.

Selection.

Table 5.4 The rates of synonymous and non-synonymous change in Kiwi Class II MHC

Area of DNA	Rate Ns	Rate S	Rate Ns/ Rate S	z test p()
NIB alleles	0.185	0.23	0.804	0.201
LSK alleles	0.186	0.229	0.812	0.245
Rowi alleles	0.175	0.235	0.745	0.089
all kiwi alleles	0.174	0.23	0.757	0.135
BS1/2/3 alpha (α)	0.253	0.329	0.769	0.228
BS 1/2/3	0.245	0.229	1.070	0.424
BS1	0.173	0.118	1.466	0.392
BS2	0.263	0.134	1.963	0.125
BS3	0.176	0		
Alpha(α) helix	0.264	0.369	0.715	0.151
rest of exon 2	0.072	0.17	0.424	0.06
264 bp seq.	0.14	0.153	0.915	0.361
267 bp seq.	0.154	0.169	0.911	0.343

Rate Ns - The number of non-synonymous substitutions per non-synonymous site from averaging over all sequence pairs. Rate S - The number of synonymous substitutions per synonymous site from averaging over all sequence pairs. Rate Ns & Rate S distances also calculated using the Pamilo-Bianchi-Li method in Mega4 (Pamilo and Bianchi 1993; Tamura et al 2007). All positions containing gaps and missing data were eliminated from the dataset. The Z tests was used to test the significance of the rate Ns/ rate S ratio i.e., if the ratio was >1 it was tested for balancing (positive) selection and if it was <1 it was tested for purifying (negative) selection. Values of P less than 0.05 are considered significant at the 5% level. The variance of the difference was computed using the bootstrap method (500 replicates) in Mega4 (Tamura et al 2007).

Recognisable polymorphic subdomains within the PBR that align with those found in the human DRβ molecule are also analysed separately - BS1, BS2, BS3 and ά helix (She et al 1991). The rest of the exon 2 refers to DNA sequence of exon 2 that are not within these polymorphic subdomains. The size of some of these areas is very small e.g. BS3 is 2 amino acid sites or 6 bp in size. It is hard to get a statistically significant result with such small sequences.

The amino acids sequences coded for by the kiwi MHC class II B exon 2 are compared to similar sequences found in other birds, an amphibian and even a human sequence. Figures 5.9, 5.11 & 5.13 show the alignments of LSK, NIB and rowi respectively. They show that the polymorphism is concentrated in comparable hypervariable regions BS1, BS2, BS3 and the ά helix as discovered by Brown (Brown et al 1993) and She (She et al 1991). This theme of the variation being concentrated at specific areas is clearly shown in Figures 5.10, 5.12 & 5.14 (LSK, NIB and rowi). The amino acid sequences of each species tend to show variation at specific codon positions and not at others. This pattern is also shown when LSK, NIB and rowi alleles are compared in Figures 5.15 & 5.16

Figure 5.9 Comparative amino acid sequence alignment for MHC class II B exon 2 sequences in the LSK. The LSK genomic DNA sequences are translated to their amino acid sequences and compared to other avian and non avian DRB type sequences from the NCBI database. Dots indicate the amino acid is identical to the LSK sequence ApLSKDRB 001 exon 2, a dash represents a space in the sequence. Recognisable polymorphic subdomains from the human DRβ molecule - BS1, BS2, BS3 and ά helix are outlined in green (She et al 1991). **Amino acids that contact the peptide in human DRβ molecule are indicated by a plus sign (+)** (Brown et al 1993).

```
                        BS 1                BS 2       BS3                                  ά helix
                      + + +                 + + +    + +         +           +     ++    +    + ++  +     +  ++  ++  ++
ApLSKDRB-1            --MNKYECQF LNGTERVRLV HRRIYNRQQL LHFDSDVGFY VADSPLGEPD AKYWNGQPDL IEQRRAEVDT VCRHNYVGVT PFTVERRG-
ApLSKDRB-2            LE.H.A...Y .........L D.Y...Q..Y V.......VF ...I...... ......LT.F V......... ......GVA. .........-
ApLSKDRB-3            LD.SIF...Y ........Y. ..N...G.LM ........V. .......... .E........ ..E..GA..R F.....GVFS .........-
ApLSKDRB-4            LD.G.T...Y ........FL E.HV...... V......... .......... .....S.TEV L.HAQNA..M F..L..GVAQ ADH.VG.TG
Penguin               QE.G.A..H. ........F. E.Y......N V.......Y. ...T...... .....S.T.F L..K...... Y.....GVG. .......V-
Chatham_Is_Robin      QW.F.G..H. I....K..Y. V.NF...EE. VR......R. .GLT.F..KQ .R...NN.A. M......... ......KVS. ..S....--
Finch                 QY.G.L..H. T....K..F. D.Y....E.F VM......EF .GVQ....KN ..RR.SN.ER M.YK.GL... Y.....RI.. ..S..A.V-
Pheasant              LHGVIF..H. V...QQ..H. E.D.H....Y A.......K. ...T....LQ .E...NNTEY M.Y..G...R Y.....E..E S...Q..V-
Blackbird             QH.Q.F..Y. I....K..YL QKY.....PI VR......H. .GFT.Y..MW ..QL.SD..I M.YQ.G...R Y.....EVFR ..IT...V-
Jay                   QE.Y.D.... I......KF. V.MF...L.Y AM......HF .GFT.Y..KQ .R.R.SL..F M.NT.TA..W Y..N..EVS. ..S....V-
Gt_reed_warbler       QE.G.V..H. I....K..F. Q.N....VED VR......HH .GFT.Y..KC .QD..SN.E. M.YK..A... ......PI.A ..S.Q..V-
Human                 LWQL.F..H. F........L E.C...QEES VR......E. R.VTE..R.. .E...S.K.. L.....A..N Y.....GV.E S...Q..V-
Salamander            VTQV.H..H. ...S....Y. V.FS..Q.PF V.....T.VF Q..T.F.V.. .....S.KEV L..A...... F..F..GIFE D.MQR..V-
```

HsCAA68171 is Human MHC Class II β haplotype DRB1 from NCBI database (unpublished).
BAE15980 Humboldt penguin (*Spheniscus humboldti*) MHC Class II β haplotype DRB1*0104 (Kikkawa et al 2005)
AAZ81957 Tiger salamander (*Ambystoma tigrinum*) MHC class II antigen beta chain from NCBI database (unpublished).
AY730416 Chatham Is black robin (*Petroica traversi*) (Miller and Lambert 2004a)
AJ224344 Ring-necked pheasant (*Phasianus colchicus*) (Wittzell et al 1999)
AJ404371 Great reed warbler (*Acrocephalus arundinaceus*) (Westerdahl et al 2000)
L42335 Bengalese finch (*Lonchura striata*) (Vincek et al 1995)
U23970 Red-winged blackbird (*Agelaius phoeniceus*) (Edwards et al 1995b)
U23975 Scrub jay (*Aphelocoma coerulescens*) (Edwards et al 1995b)

Figure 5.10 Number of alternative amino acids for each position in exon 2 of the LSK Alleles.

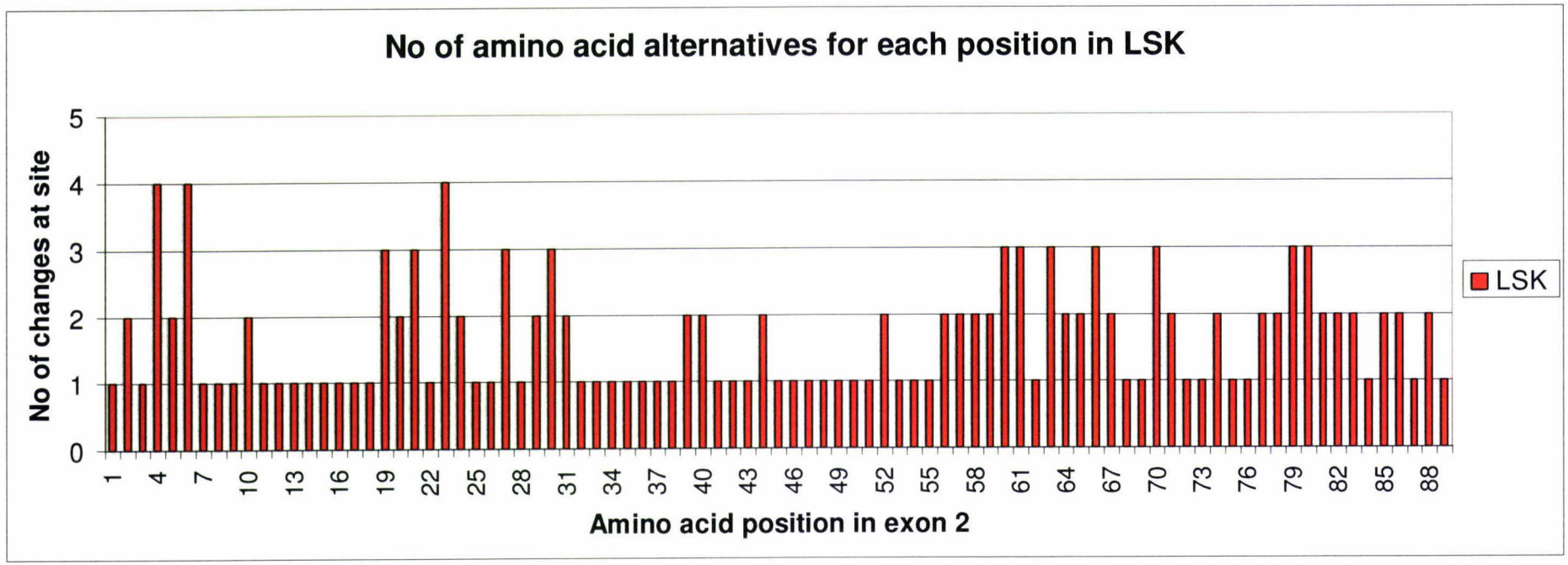


Figure 5.11 Comparative amino acid sequence alignment for MHC class II B exon 2 sequences in the NIB. Sequences are translated from NIB genomic DNA and compared to other avian and non avian DRB type sequences from the NCBI database. See the bottom of Figure 5.9 for the NCBI Accession number. Dots indicate the amino acid is identical to the to the NIB sequence Apma 1, a dash represents a space in the sequence. Recognisable polymorphic subdomains from the human DRβ molecule - BS1, BS2, BS3 and ά helix are outlined in green (She et al 1991). Amino acids that contact the peptide in human DRβ molecule are indicated by a plus sign (+)(Brown et al 1993).

```
                      BS 1                BS 2       BS3                                  ά helix
                     + + +                + + +     + +         +          +     ++    +   + ++  +     +  ++  ++  ++
Apma 001             LAMHKEECQY LNGTERVRLL DRYIYNRQQI VHFDSDVGFY VADIPLVEPD AKYWNGQPDI IEQKQAEVDT VCRHNYGVGT PFTVERRG-
Apma 002             .E.G.A.... .......... ......Q..Y ........VF ...S..G... ......LT.F ..R.R..... ........AQ ADH.VG.TG
Apma 003             .E.G.S.... ........F. ....H....L L.......V. A..S..G..L ..A..S..E. L.RA.NA... IS...SW.FS ........-
Apma 004             .E.G.S.... ....K...F. V...H....F ........VF ...S..G... .....S..EV LAHA.NAL.M F..CS.E.AQ ADH.VG.TG
Apma 005             .E.G.S.... ........F. ....H....L L.......V. A..S..G..L ..A..S..E. L.RA.NAM.. IS....W.FS ........-
Apma 006             .......... .......... .......... .......... .......... .......... .........A. ........-
Apma 007             .E.N.G.... ........FV H.N...G.LM L.......V. ...S..G... .....S...F ....RGA... Y.......AQ ADH.VG.KG
Apma 008             .....A.... .....Q..F. ....H....L .......... ......G... .E.......L ..E.R.SENR F.......AQ ADH.VG.RG
Apma 009             .Q.F.A...F .....Q..YV A.S......D ........VF ...S..G... .....S...L ..ERR.K... Y.......V. ........-
Apma 010             .E.G.S.... .....Q...V Q.N...G.LM L.......V. ..NS..G... .....S...L ..DRR..... F..CS.V.VQ ADH.VG.TG
Apma 011             .E...G.... .......... E........Y ........VF ...S..G..S .....S...L ..DRR.A... F.....VGV. ........-
Penguin              QE.G.A..HF ........FV E........N ........Y. ...T..G... .....S.T.F L...R..... Y......... .......V-
Chatham_Is_Robin     -W.F.G..HF I....K..YV V.NF...EEL .R......R. .GLT.FG.KQ .R...NN.AL M..RR..... ......K.S. ..S....--
Finch                QY.G.L..HF T....K..FV .......E.F .M......EF .GVQ..G.KN ..RR.SN.ER M.Y.RGL... Y.....RIV. ..S..A.V-
Pheasant             .HGVIF..HF V...QQ..HV E.D.H....Y A.......K. ...T..G.LQ .E...NNTEY M.YRRG...R Y.....EGVE S...Q..V-
Blackbird            QH.Q.F..YF I....K..Y. QK......P. .R......H. .GFT.YG.MW ..QL.SD... M.YQRG...R Y.....E.FR ..IT...V-
Jay                  QE.Y.D...F I......KFV V.MF...L.Y AM......HF .GFT.YG.KQ .R.R.SL..F M.NTRTA..W Y..N..E.S. ..S....V-
Gt_reed_warbler      QE.G.V..HF I....K..FV Q.N....VED .R......HH .GFT.YG.KC .QD..SN.EL M.Y.R.A... ......PIVA ..S.Q..V-
Human                .WQL.F..HF F......... E.C...QEES .R......E. R.VTE.GR.. .E...S.K.L L..RR.A..N Y.......VE S...Q..V-
Salamander           VTQV.H..HF ...S....YV V.FS..Q.PF ......T.VF Q..T.FGV.. .....S.KEV L..AR..... F..F...IFE D.MQR..V-
```

Figure 5.12 Number of alternative amino acids for each position in exon 2 of the NIB Alleles.

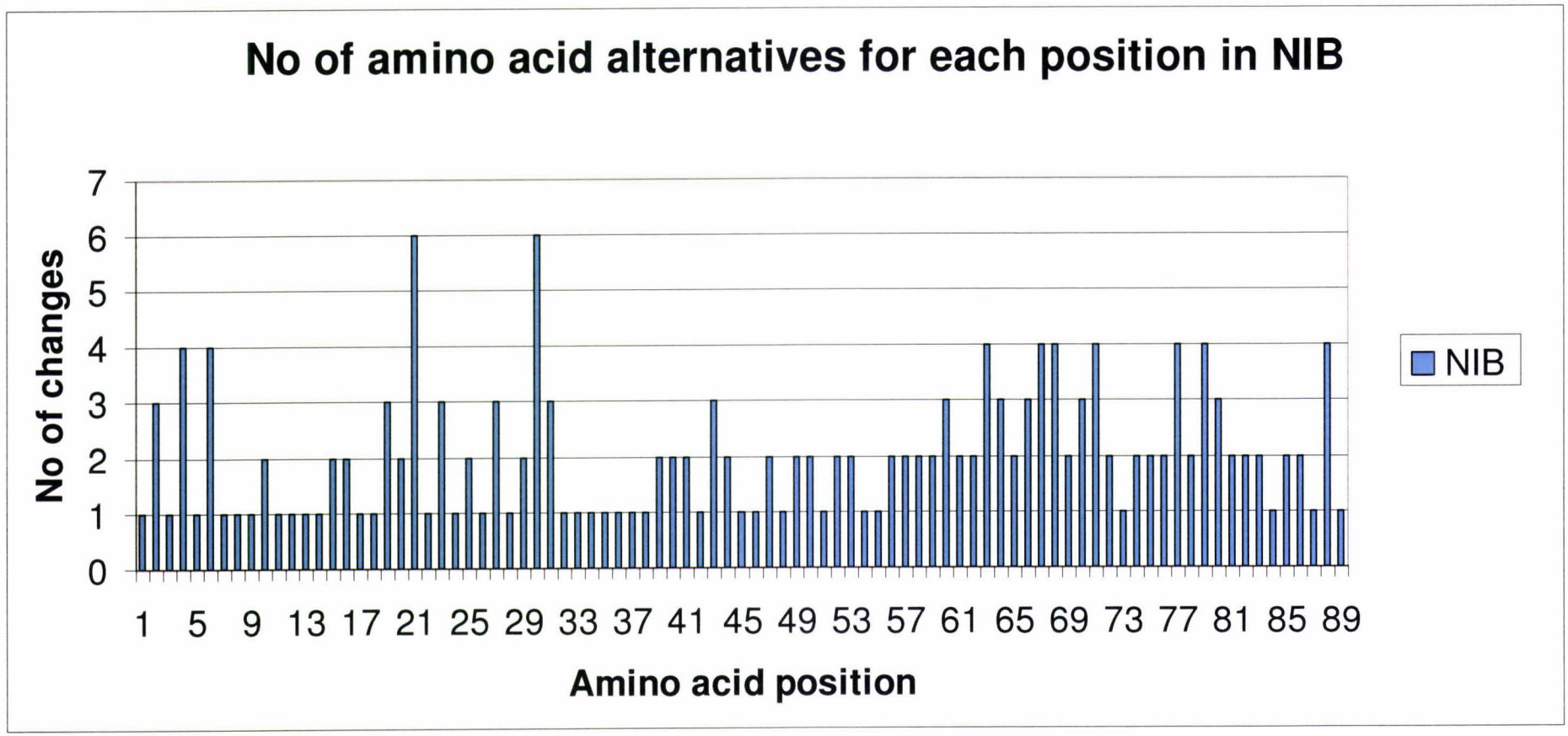


Figure 5.13 Comparative amino acid sequence alignment for MHC class II B exon 2 sequences in the ROW. Sequences are translated from Rowi genomic DNA and compared to other avian and non avian DRB type sequences from the NCBI database. See the bottom of Fig.5.9 for the NCBI Accession number. Dots indicate the amino acid is identical to the Rowi sequence Apro 001, a dash represents a space in the sequence. Recognisable polymorphic subdomains from the human DRβ molecule - BS1, BS2, BS3 and ά helix are outlined in green (She et al 1991). Amino acids that contact the peptide in human DRβ molecule are indicated by a plus sign (+)(Brown et al 1993).

```
                      BS 1                BS 2       BS3                                  ά helix
                    + + +                 + + +     + +         +          +      ++  +    + ++  +     +  ++  ++  ++
Apro 001            LEVNKGECQY LNGTERVRFV HRNIYNGQLM LHFDSDVGVY VADSPLGEPD AKYWNSQPDF IEQKRGAVDT YCRHNYGVAQ ADHVVGRKG
Apro 002            ..MG.S.... .........L V.Y.H.R.QF V....E...F ...I..... ........EV LAHAQN.L.. FS.CS.E... .....S.P.
Apro 003            .AMH.E.... ........LL D.Y...R.QI V.......F. ...I..V... .....G.T.I ....QAE... V........T PFT.ER.G-
Apro 004            ..MH.A.... .........L D.Y...Q.QY V........F .......... .....GLT.. V..R.AE... V........T PFT.ER.G-
Apro 005            ..MF.F.... .........L D.Y.H.R.QF V.......F. .......... .........L ..EE.AS... ........VT PFT.ER.G-
Apro 006            .AMH.A.... .....Q...L D.Y.H.R.QF V.......F. ...L..... .....G...L ..E..VSENR F........T PFT.ER.G-
Apro 007            ..MG.S.... .........L D.Y.H.R.QY V.......F. .........N .......... ...R..... .......... .......T.
Apro 008            ..MD.F.... .......... .......... ....N...F. .......... .....G...I ...R.AE... V.......FS PFTMER.G-
Apro 009            .QMF.A...F .....Q..Y. A.S...R.QD V........F .......... .........L ..ER.AK... .......... .......R.
Apro 010            ..MH.S.... V....Q.KY. E.YL..R.QY V...C.L... .........T .........L ..E.QAK... ........VT PFT.ER.G-
Apro 011            ..MH.A.... .........L D.Y...Q.QY V........F .......... .....GLT.. V..R.AE... V........T PFT.ER.G-
Apro 012            ..ML.A.... .......... V...H.R.QF V......... .........T ........EV LAHAQN.... F......... .........
Apro 013            ..MH.S.... V....Q..Y. E.Y.H.R.QL V...C.L... .......... .........I ..E..AK... ........DA PFT.ER.G-
Apro 014            ..MD.F.... .......... .......... ....N...F. .......... .........I ...R.AE... V.......FS PFTMER.G-
Penguin             Q.MG.A..HF .......... E.Y...R.QN V.......Y. ...T...... .......T.. L....AE... ........GT PFT.ER.V-
Chatham_Is_Robin    -WMF....HF I....K..Y. V..F..REEL VR......R. .GLT.F..KQ .R...NN.AL M..R.AE... V.....K.ST PFS.ER.--
Finch               QYMG.L..HF T....K.... D.Y...REQF VM......EF .GVQ....KN ..RR..N.ER M.Y...L... ......RIVT PFS.EA.V-
Pheasant            .HGVIF..HF V...QQ..H. E.D.H.R.QY A.......K. ...T....LQ .E...NNTEY M.YR..E..R ......EGVE SFT.QR.V-
Blackbird           QHMQ.F..YF I....K..YL QKY...R.PI VR......H. .GFT.Y..MW ..QL..D..I M.YQ..E..R ......E.FR PFITER.V-
Jay                 Q.MY.D...F I......K.. V.MF..RLQY AM......HF .GFT.Y..KQ .R.R..L... M.NT.T...W ...N..E.ST PFS.ER.V-
Gt_reed_warbler     Q.MG.V..HF I....K.... Q.....RVED VR......HH .GFT.Y..KC .QD...N.EL M.Y..A.... V.....PIVA PFS.QR.V-
Human               .WQL.F..HF F.......LL E.C...QEES VR......E. R.VTE..R.. .E.....K.L L..R.A...N ........VE SFT.QR.V-
Salamander          VTQV.H..HF ...S....Y. V.FS..Q.PF V.....T..F Q..T.F.V.. .......KEV L..A.AE... F..F...IFE DFMQRR.V-
```

Figure 5.14 Number of alternative amino acids for each position in exon 2 of the Rowi Alleles.

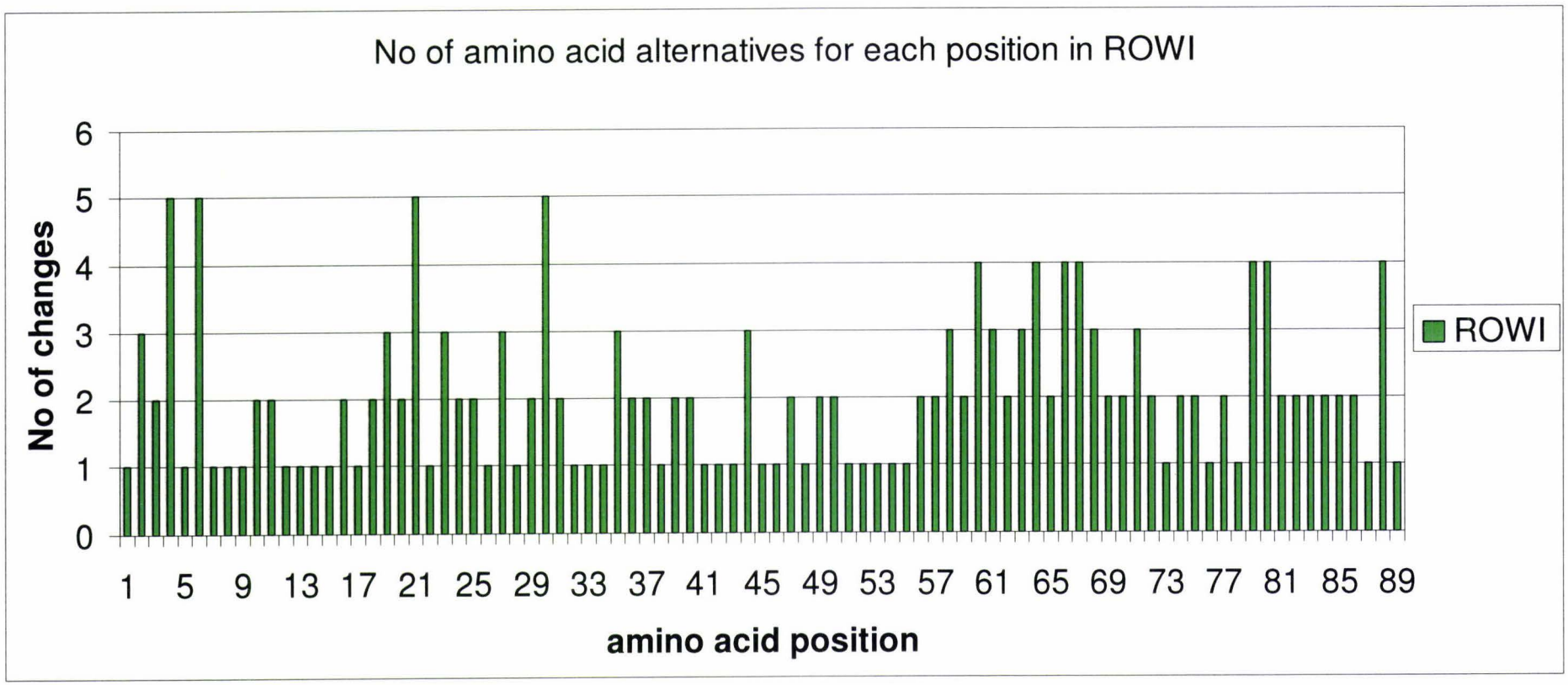
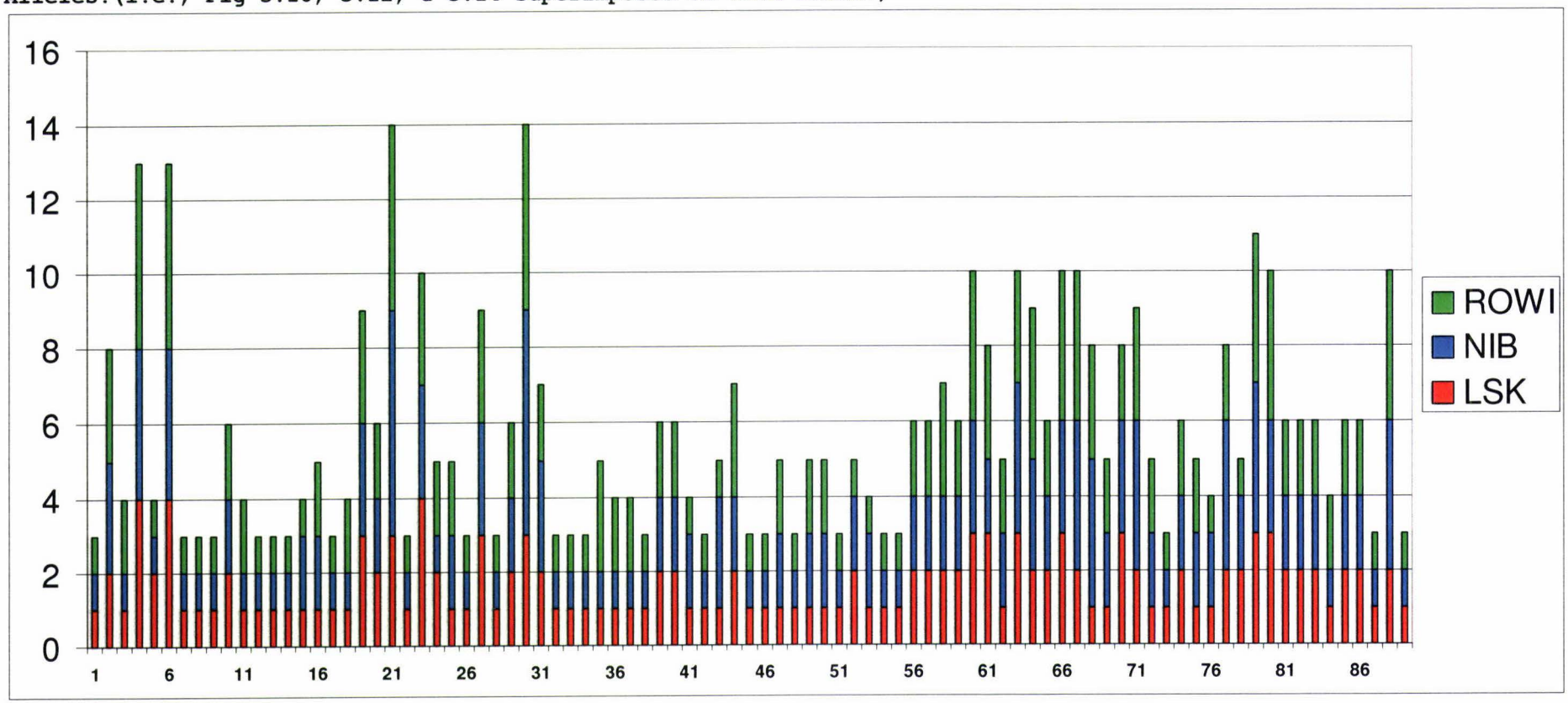


Figure 5.15 Comparative amino acid sequence alignment for MHC class II B exon 2 sequences in the NIB, LSK and Rowi. The Sequences are translated from genomic DNA and compared to each other. The layout is the same as Fig 5.9, 5.11 & 5.13. The prefix Apro shows Rowi amino acid sequences. Apow is for LSK amino acid sequences and Apma is for NIB.

```
                BS 1                BS 2       BS3                                  α helix
              + + +                 + + +     + +         +          +    ++   +   + ++  +     +  ++  ++  ++
Apro 001      LEVNKGECQY LNGTERVRFV  HRNIYNGQLM LHFDSDVGVY VADSPLGEPD AKYWNSQPDF IEQKRGAVDT YCRHNYGVAQ ADHVVGRKG
Apro 002      ..MG.S.... .........L V.Y.H.R.QF V....E...F ...I...... ........EV LAHAQN.L.. FS.CS.E... .....S.P.
Apro 003      .AMH.E.... ........LL D.Y...R.QI V........F. ...I..V... .....G.T.I ....QAE... V.........T PFT.ER.G-
Apro 004      ..MH.A.... .........L D.Y...Q.QY V........F .......... .....GLT.. V..R.AE... V.........T PFT.ER.G-
Apro 005      ..MF.F.... .........L D.Y.H.R.QF V.......F. .......... .........L ..EE.AS... ........VT PFT.ER.G-
Apro 006      .AMH.A.... .....Q...L D.Y.H.R.QF V.......F. ...L...... .....G...L ..E..VSENR F.........T PFT.ER.G-
Apro 007      ..MG.S.... .........L D.Y.H.R.QY V.......F. .........N .......... ...R...... .......... .......T.
Apro 008      ..MD.F.... .......... .......... ....N...F. .......... .....G...I ...R.AE... V.......FS PFTMER.G-
Apro 009      .QMF.A...F .....Q..Y. A.S...R.QD V........F .......... .........L ..ER.AK... .......... .......R.
Apro 010      ..MH.S.... V....Q.KY. E.YL..R.QY V...C.L... .........T .........L ..E.QAK... ........VT PFT.ER.G-
Apro 011      ..MH.A.... .........L D.Y...Q.QY V........F .......... .....GLT.. V..R.AE... V.........T PFT.ER.G-
Apro 012      ..ML.A.... .......... V...H.R.QF V......... .........T ........EV LAHAQN.... F......... .........
Apro 013      ..MH.S.... V....Q..Y. E.Y.H.R.QL V...C.L... .......... .........I ..E..AK... ........DA PFT.ER.G-
Apro 014      ..MD.F.... .......... .......... ....N...F. .......... .........I ...R.AE... V.......FS PFTMER.G-
Apow 001      --M..Y...F ........L. ..R...R.QL ........F. .......... .....G...L ...R.AE... V.....VGVT PFT.ER.G-
Apow 002      ..MH.A.... ........LL D.Y...Q.QY V........F ...I...... .....GLT.. V..R.AE... V.........T PFT.ER.G-
Apow 003      .DMSIF.... ........Y. .......... .......... .......... .E...G...L ..ER.....R F.......FS PFT.ER.G-
Apow 004      .DMG.T.... .........L E.HV..R.QL V.......F. .......... .......TEV L.HAQN...M F..L...... .......T.
Apma 001      .AMH.E.... ........LL D.Y...R.QI V.......F. ...I..V... .....G...I ....QAE... V.......GT PFT.ER.G-
Apma 002      ..MG.A.... ........LL D.Y...Q.QY V........F .......... .....GLT.. ..R..AE... V......... .......T.
Apma 003      ..MG.S.... .........L D.Y.H.R.QL .......... A........L ..A.....EI L.RAQN.... IS...SW.FS PFT.ER.G-
Apma 004      ..MG.S.... ....K....L V.Y.H.R.QF V........F .......... ........EV LAHAQN.L.M F..CS.E... .......T.
Apma 005      ..MG.S.... .........L D.Y.H.R.QL .......... A........L ..A.....EI L.RAQN.M.. IS....W.FS PFT.ER.G-
Apma 006      .AMH.E.... ........LL D.Y...R.QI V.......F. ...I..V... .....G...I ....QAE... V.........T PFT.ER.G-
Apma 007      ..M....... .......... .......... .......... .......... .......... .......... .......... .........
Apma 008      .AMH.A.... .....Q...L D.Y.H.R.QL V.......F. ...I...... .E...G...L ..E..ASENR F......... .......R.
Apma 009      .QMF.A...F .....Q..Y. A.S...R.QD V........F .......... .........L ..ER.AK... ........VT PFT.ER.G-
Apma 010      ..MG.S.... .....Q..L. Q......... .......... ..N....... .........L ..DR.AE... F..CS.V.V. .......T.
Apma 011      ..MH...... ........LL E.Y...R.QY V........F .........S .........L ..DR.A.... F.....VGVT PFT.ER.G-
```

Figure 5.16 Number of alternative amino acids available for each position of exon 2 found in LSK, NIB & Rowi Alleles.(i.e., Fig 5.10, 5.12, & 5.14 superimposed on each other.)



Discussion.

The sequences derived from the Class II B exon 2 primers, KiwintA1F & KiwintA2R showed a variety of putative alleles and pseudogenes, as well as two sizes of sequence (281 & 284 bp). Examining the exon 2 sequences revealed it came in three sizes (not including pseudogenes): 258 bp (1 allele), 264bp (16 alleles) and 267bp (12 alleles). Pseudogenes are commonly found in mammalian MHC and attributed to a birth and death of genes model operating (Klein et al 1993b; Nei et al 1997). Pseudogenes have been identified in both class I & II of passerine MHC (Edwards et al 2000; Hess et al 2000) but are absent in chickens and not commonly found in other avian groups (Kaufman et al 1999; Ekblom et al 2003; Alcaide et al 2007). Suspected pseudogenes have been identified in the NIB (2) and the Rowi (3), but not the LSK.

Although the development work on the KiwiA1F & KiwintA2R primers was performed on NIB DNA, a large number of "alleles" have been detected in the Rowi population. This is consistent with "concerted evolution" and transpecies polymorphism acting on these sites. Some researchers consider avian MHC shows more signs of "concerted" evolution while mammalian MHC follows a more divergent pattern of evolution (Edwards et al 1999; Wittzell et al 1999). Concerted evolution has been proposed for multigene families (like MHC) with member genes being homogenised by unequal cross over or gene conversion (Ohta 1984). This results in the related genes evolving together as though constituting a single locus. However the pattern seen in birds could be obtained by concerted evolution or post-speciation duplication (Edwards et al 1999; Hess and Edwards 2002).

The number of loci amplified in each species by these primers appears to be greater than one but the exact number is uncertain. This is not unexpected in avian MHC which appears to be characterized by multiple, closely related loci, although placing the primers in the introns was expected to reduce the likelihood of amplifying multiple loci (Edwards et al 1995a; Hess and Edwards 2002; Miller and Lambert 2004a). It has been suggested that care should be taken when inferring loci/alleles from exon 2 sequences alone (Miller and Lambert 2004b). The results (see Figures 5.2-5.4) do suggest that there are three loci in NIB; three (two possibly) loci in LSK; and five (possibly six) loci

in Rowi. In comparison to similar avian MHC Class IIB results, Alcaide et al (2007) found in birds of prey evidence of small numbers of loci (1-3 genes) while some passerine may have up to six loci (Sato et al 2001).

The putative gene conversion events identified in Table 5.3 are relatively small fragments ranging from 43 -110 bp. However as only the small exon 2 (267 and 264 bp) was analysed, larger gene conversion fragments with sections outside of exon 2 may have occurred. The putative gene conversion events appear to be spread across the tree, which is consistent with random occurrence. The gene conversion event between Apma 008 and Apma 003 and Apma 005, may have predated the divergence of Apma 003 and Apma 005 as the section translocated is identical i.e., a 43bp section from position 48 to 90.

This investigation of Kiwi MHC supports the suggestion that avian MHC sequences evolve by concerted evolution with genetic conversion a likely cause. The putative gene conversion events (Table 5.3 & Fig 5.6) are across all three species of kiwi. The LSK showed a single gene conversion event between Apow 001 and Apow 004. In Rowi, four such events showed when no mismatches (mutation after a gene conversion event) were allowed but six gene conversions were possible when a limited amount of mutation after a gene conversion event were considered. In NIB five gene conversion events may have occurred between putative alleles. The minimum number of recombination events (R_M) estimated by DnaSP (Rozas et al 2003), using the exon 2 coding sequences suggested a much higher amount of activity (LSK = 7 , NIB = 14, Rowi = 20).

Most PBR codons show higher non-synonymous substitution rates (rate Ns) compared to the rate of synonymous substitution (rate S) in classical Class I & II MHC i.e., rate Ns>rate S and this is often attributed to balancing selection (reviewed in (Hughes and Nei 1988; Potts and Slev 1995; Hughes and Yeager 1998b; Hedrick 1999; Bernatchez and Landry 2003). Over time there is an accumulation of mutations that change the amino acid structure of the groove (PBR) making a functional difference compared to those DNA changes that are silent and do not alter the expressed structure (Hughes and Nei 1988; Hughes and Nei 1989a; Hughes et al 1994)(reviewed (Sommer 2005)).

Identified within the PBR of the human DRβ molecule (Class II HLA) are polymorphic subdomains called BS1, BS2, BS3 and ά helix, which often contain higher levels of polymorphism(She et al 1991). The alignment of variability in the kiwi codons and these polymorphic subdomains can be seen in Figures 5.9, 5.11 & 5.13. Finding a rate Ns<rate S ratio (Table 5.4) for not only each kiwi species (LSK, NIB & rowi) but also for the ά helix subdomain was an unexpected result. However, it is noteworthy that none of the results (Table 5.4) were statistically significant under the z test. A result of rate Ns<rate S is typical of many other functional genes as non synonymous changes disrupt a protein's structure and are removed by conservative or purifying selection (Hughes et al 1994). When a similar result (rate Ns<rate S) was found in bank voles (*Clethrionomys glarelus*) and Hawaiian honeycreepers *(Drepanidinae*) it was attributed to isolating several separate clusters of alleles, some of which do not appear to be operating as classical class II MHC (Jarvi et al 2004; Axtner and Sommer 2007). Although it is already suspected that this process recovered alleles from multiple loci, no clear basis for division of alleles into specific loci has been identified. Usually the analysis of the ratio of non-synonymous to synonymous changes (rate Ns/rate S) is performed on alleles of the same loci, though it has been used before in multiple loci in avian MHC (Miller and Lambert 2004b). The magnitude of balancing selection, as measured by rate Ns/rate S can be underestimated when using divergent sequences (i.e. interlocus comparisons) and where there is saturation of non-synonymous changes (Takahata et al 1992; Edwards et al 1998). Garrigan and Hedrick (Garrigan and Hedrick 2003) also suggested caution in using of rate Ns/rate S (i.e. the neutrality test) alone to evaluate selective forces. MHC may also suffer from the signals of selection being generated in a relatively short period of microevolutionary time, yet taking exceptionally long periods of time to be erased in the absence of selection (Garrigan and Hedrick 2003). The division of the 264 & 267 bp sequences as the earliest split is found in both rowi and LSK in Figures 5.2 & 5.4, but is not continued in NIB. Although the tendency for all the kiwi allele in combined analysis (Fig 5.5 & Fig 5.6) showed a 264/267 split. Even when the two sizes of allele are analysed separately the ratio is still rate Ns<rate S (0.915, 0.911), and still not statistically significant. These results are a snapshot in evolutionary history, and at best any interpretation will have many underlying assumptions e.g., as we lack knowledge about the affiliation of alleles to

loci, and it is impossible to account for linkage disequilibrium. Having said that these results suggest either:

A/ Some or all of these alleles are from non classical MHC loci, possibly similar to those found on the chickens Rfp-Y fragment. If some are not expressed or are non functional, then rate Ns/rate S would be expected to tend towards rate Ns=rate S (rate Ns/rate S =1) as neutral evolution predominated. However after a duplication event, it has been suggested new locus may undergo "sub- or neo-functionalisation" which could impose a new purifying selection force preventing them from diverging (reviewed(Zhang 2003; Nei 2005)), and this may account for rate Ns<rate S.

B/ Although the BS1 & BS2 areas appear to be under balancing selection the remainder of the exon appears to under a negative (purifying) selection. It could be speculated an infectious disease may be involved, but more direct evidence would be required.

The position of the amino acid changes in all the alleles are compared within and between species (Figures 5.10, 5.12, 5.14 & 516). This comparison included all the putative alleles for each species although some alleles are 88 and some 89 amino acids long, and one LSK allele which are 86 amino acids long. The amino acid alignments show the differences between exon 2 alleles are concentrated at particular positions. The positions affected by the difference in allele length are the last few amino acids position. The concentration of changes are shown to align with similar hypervariable regions found in Human MHC Class II β haplotype DRB1, others birds and salamander in Figures 5.9, 5.11, & 5.13. This alignment of polymorphic sites in the three kiwi species coincides with the polymorphic subdomains - BS1, BS2, BS3 and ά helix (She 1991) and the contact points indicated by a plus sign (+) identified in the human DRβ molecule (Brown et al 1993). This would imply the Kiwi alleles are structurally and functionally similar to the HLA – DRB. The number of changes at each amino acid site in each species is shown in Figure 5.16. This (Fig 5.16) confirms the trend found that the three species of Kiwi mainly accumulate polymorphism at the same sites in PBR, and some of these sites tend to accumulate more polymorphic than others (BS1,BS2 >α helix).

The Little Spotted Kiwi is considered at low risk of extinction, as long as Kapiti Island, and the other five sanctuaries remain free of mammalian predators (Robertson and Colbourne 2004). However these results indicate the population on Red Mercury may have limited MHC diversity, and hence may be more at risk than previously suspected. The results show four different alleles in the eight birds, however two of the alleles were found in all eight birds. The other two alleles were present in seven and four birds respectively i.e., there was not a large amount of variation found between birds in this population. This lack of variation looks to be severe considering multiple loci appear to be examined i.e., a lack of polymorphism at several loci.

Minimal MHC diversity in the LSK is consistent with a population that has been through a severe bottleneck. The once abundant LSK showed a decline to near extinction in the twentieth century with initially only two offshore island populations remaining. There have been many LSK translocations and there are currently six populations on islands (five islands, one mainland island) that could also be affected by founder and bottleneck events. The presence of limited Class II DRB haplotypes in the LSK's does not rule out more extensive polymorphism elsewhere in it's MHC. Cotton-top tamarins (*Saguinus oedipus*) showed limited polymorphism in class I MHC and several areas of Class II MHC (DQA1, DQB1, DQB2) but extensive polymorphism in four different DRB loci (Gyllensten et al 1994). However, work on the LSK at other DNA sites also showed limited variation (Shepherd 2006).

Recent cytochrome b analysis showed only a single haplotype in today's Kapiti Is LSK (Shepherd 2006). LSK from Kapiti Is have been used in relatively small numbers to start populations on other island i.e. the founder numbers may be small enough to be their own bottleneck. If small founder populations are used or genetic bottlenecks develops, genetic restoration similar to that seen in the Florida panther (Hedrick 2005)may help increase genetic diversity and limit genetic drift, but it is not without its own risks(Tallmon et al 2004). The LSK populations have prospered in the short term when predators are removed, however concern is being raised about loss of genetic diversity and resulting decreased fitness in many NZ birds (Jamieson et al 2006). Tompkins (2006) when examining inbreeding depression showed a decrease in immune competency in some NZ parakeets and suggested hybridisation as a possible solution. If

the lack of genetic diversity found in the LSK on Red Mercury Island is also present on Kapiti Island, then it may be necessary to give consideration to hybridisation as a source of genetic diversity. This suggestion is likely to stimulate a lively debate as to there are many pro's and con's of such and action and it also will focus attention onto how important is polymorphism of the MHC in declining populations?

The Okarito Kiwi Sanctuary is the home of the only known rowi population estimated at ~ 250. Interestingly enough, mitochondrial DNA and allozyme analyses shows rowi are more closely related to the North Island Brown than the Tokoeka (South Island Brown kiwi) (Herbert and Daugherty 2002; Burbidge et al 2003). The amount of genetic diversity in the MHC was not consistent with a population that has been through a severe bottleneck recently. Analysis of ancient DNA by Shepherd (2006) shows a greater distribution in the past for rowi, and is consistent with this being a remnant population. This raises the possibility of undiscovered rowi populations existing in areas like the north-west of the South Island and even possibly the south-east of the North Island where it may previously have roamed.

The presence of 11 suspected alleles in 10 NIB birds would imply despite Westerdahl (2000) suggestion that non-migratory birds could have lower levels of MHC polymorphism this NIB population has a reasonably polymorphic MHC. Analysis of NIB mitochondrial DNA showed the highest level of structuring seen in a bird population with almost every population having its own private haplotypes (Baker et al 1995; Burbidge et al 2003). Shepherd (Shepherd 2006) showed with ancient DNA analysis of kiwi that this high level of structuring also existed in the past together with even more genetic variation. This level of DNA structuring in populations is more commonly associated with mammals. The level of structuring in NIB populations may extend to the MHC which leads to the question: Are there "new" haplotypes to be discovered in other NIB populations e.g., Kiwi on Little Barrier Kiwi or the East Cape? Certainly the presence of alternative MHC polymorphism in other NIB populations would allow the introduction of more diversity to fragmented and isolated populations by judicious transfer of birds.

Chapter 6

Summary and Discussion of Future Work

6.1 Synopsis of Major Findings

The major findings of this thesis are summarised below in relation to the research aims (*italicised*):

To be able to reliably amplify and characterise a Class II B Protein Binding Region in the Kiwi Major Histocompatibility Complex (MHC) :

A pair of primers (KiwiA1F & KiwintA2R), was designed to amplify the Class II B Protein Binding Region (exon 2) of the North Island Brown Kiwi, Little Spotted Kiwi, and Rowi. These primers are highly likely to also amplify sequences in Great Spotted Kiwi and Tokoeka, as they do so in other spotted and brown kiwis. Various modifications were performed at each stage after DNA extraction to minimise the production of artefact with the use of SURE®2 clones and TempliPhi being the most successful approach.

To compare and analyse the MHC polymorphism found in three different Kiwi species and populations:

The eight Little Spotted Kiwi (LSK) from Red Mercury Island showed four different alleles, with most birds containing all four alleles. This shows little variation in the population and is consistent with the genetic bottlenecks in their recent history. The result does raise concern that although the LSK have rebounded in numbers in the last few years, they may be "at risk" to an epidemic and consideration should be given to conservation management strategies for enhancing their MHC diversity. The rowi population although currently small showed 14 alleles in 18 birds and three suspected pseudogenes. This result is consistent with the rowi once being a larger population, possibly spread from its current site up the north-east of the South Island to the south-west of the North Island. The 12 North Island Browns showed 11 alleles and two possible pseudogenes. The NIB sample come from near Whangarei which is part of the large Northland population and does not appear to lack polymorphism. Neither the rowi nor the NIB kiwi showed the limited amount of alleles seen in the LSK

To compare and contrast the Class II B PBR with other avian and mammalian sequences:

The sequences found in all three kiwi species were similar to Class II DRB like genes with similar patterns of amino acid substitution sites to humans and other avian and mammalian species. Analysis of the DNA sequences supports the process of concerted evolution with gene conversion similar to other avian MHC as occurring in the kiwi. However the presence of putative pseudogenes, although found in passerines is more associated with the mammalian MHC and the reputed divergent evolution process of mammalian MHC under a birth and death model (Ota and Nei 1994; Hughes and Hughes 1995).

In this thesis it has also been shown:

A/ The primers developed in this thesis, KiwiA1F & KiwintA2R, produced two different sized sequences in the three populations, 281 & 284bp long. The 284 bp sequence contained a 267 bp long exon 2 sequence, while the 281 bp sequence produced 258 bp and 264 bp long exon 2 sequences.

B/ The different sequences that fit the criteria developed to exclude laboratory artefacts show 4 putative alleles in the LSK, 11 putative alleles in the NIB and 14 putative alleles in the Rowi. Checking exon 2 for conserved domains against the NCBI database showed significant alignment with MHC Class II B (Geer et al 2002; Marchler-Bauer and Bryant 2004). The sequences when compared by BLAST software on the NCBI database showed significant alignment with avian MHC Class II DRB – like sequences (Wheeler et al 2007). Suspected pseudogenes were also identified in the NIB (2) and the Rowi (3).

C/ Analysis of the sequence by Geneconv (gene conversion), Splits4tree (network analysis) and DnaSp (R_M) support the occurrence of multiple gene conversion and recombination events. having lead to the similarities seen in the sequences now.

D/ The strength of selection acting on the PBR region (exon 2) as measured by rate non-synonymous change over the rate of synonymous change ratio, did not show a significant effect of selection for polymorphism as found in most MHC PBR regions. When analysed at the proposed BS1 and BS2 hypervariable regions the rate non-synonymous change over the rate of synonymous change ratio did show positive selection for polymorphism but it was not statistically significant (Z test probability >0.05). Analysis of the different areas of the PBR (exon 2) did show variation in the selection found, but not at a statistically significant level.

6.2 MHC Polymorphism.

It is beyond the scope of this thesis to prove or disprove how important MHC polymorphism is for conservation of declining populations. O'Brien (O'Brien et al 1985; Brown et al 1994) maintains the cheetah shows that lack of polymorphism at the MHC increases a population's risk of debilitating disease from pathogens, however other causes for the decline of the cheetah have also been established (Caro and Laurenson 1994; Munson et al 2005). The ability of an infectious disease to drive a species to extinction in the wild has not been demonstrated yet (de Castro and Bolker 2005). For example unless a pathogen has other hosts maintaining it, when a vertebrate species gets very low in numbers the opportunity to pass on an infection is greatly limited. Lack of polymorphism in general as a result possibly of inbreeding can affect a population's ability to survive (Hedrick and Kalinowski 2000). Lack of polymorphism at the MHC in particular, intuitively suggests a greater risk to a population from endemic and emerging pathogens. This perspective leads to the question of how much polymorphism is enough? Or as a conservationist may fear, "Selection may call but there may be no mutations to answer" pg 92 (Lewontin 2000). However is a focus on polymorphism of the MHC enough? To quote H L Menken "To every complex problem there is a simple solution and it is usually wrong". Recognising the complex nature of MHC polymorphism and conservation of species, it follows the answer is not in simply MHC polymorphism alone. The host – pathogen relationship is complex and co-evolving, and in gnathostomes MHC is one part of the dynamic. Conservation management of endangered species therefore rests with approaches designed for complex systems like the six principals for parasite management proposed by Horwitz and Wilcox (2005). A multipronged strategy based on better problem definition and better tools for assessing and monitoring environmental and conservational issues is needed to ensure the survival of endangered species(Aguirre et al 2002). Unfortunately if promoting MHC polymorphism is an appropriate conservation strategy and we act upon it too late we risk losing populations and even species to extinction. It may prove safer for conservation to utilise existing MHC polymorphism in combination with other factor like mean kin relationship in maintaining genetic diversity in endangered species like the kiwi, before empirical evidence is established.

6.3 Future Work

The results of this thesis suggest further investigations are required into:

6.3.1 Apply these primers and techniques to evaluate MHC polymorphism in various kiwi populations throughout New Zealand. The procedure will need to be initially validated in Great Spotted Kiwi and Tokoeka especially the Haast kiwi. Once the type and distribution of MHC haplotypes in given populations is established, it can be used to make more informed conservation management decisions e.g. in translocating breeding birds to depauperate populations to maximise MHC polymorphism. It is of particular importance to assess the amount of polymorphism in the Kapiti Island and remains of the D'Urville Island. LSK, as they may be depauperate as the Red Mercury Island population. If the Kapiti Island population shows similar variation to the Red mercury Is. population, then there is increased importance of finding and identifying the MHC haplotypes of the D'Urville Is descendents, as they maybe a possible source of variation.

6.3.2 It appears these primers amplify more than one locus; designing primers or techniques that are specific to one locus, if possible, would enable more precise analysis of the data. Currently work on the kiwi genome is underway; data from this will enable more specific design of primers and understanding of the structure and organisation of the Major Histocompatibility Complex. The use of DGGE or SSCP instead of sequencing would make the analysis of large numbers more cost effective. Alternatively using the 3′ untranslated region of cDNA may be effective (Miller and Lambert 2004b)in amplifying a single locus.

6.3.3 We need to explore the possible use of monitoring changes in MHC haplotype frequencies in wildlife populations as a surveillance system for diseases e.g., a relationship between a cancer and free ranging California sea lions (*Zalophus californianus*) (Bowen et al 2005). The use of non invasive DNA samples like feathers and hair when discarded by the animal would be much less stressful than handling individual animals. It is difficult to investigate infectious disease in wild populations, but monitoring for dramatic changes or trends over time might indicate an infectious disease problem. Bowen (2006) has suggested examining the expression levels of DRB MHC in a population may provide a monitor of current immune activity in a population. Problems with examining genetic or expressed MHC alone include; diseases are often multifactorial, genetic resistance or sensitivity to the disease may not be

located in the MHC and balancing selection may interfere with the observable response. A potential examination of this method may be found in neonates of many species which are particularly susceptible to many parasites. Changes in haplotype frequencies from those born to those that attain adulthood might show if this is a feasible technique or patterns in expression levels over their early life may provide information on pathogen activity. This approach may not be attributable to a specific pathogen, but could be an effective monitoring technique especially for cryptic animals like kiwi. If an infectious disease was causing an epidemic in kiwi and susceptible and resistant alleles could be identified it could help with management issues by identifying the more at risk animals/populations. It has been suggested that New Zealand avifauna has been adversely affected by introduced pathogens (Meyers 1923; Moon 1988). Improving ancient DNA retrieval techniques may allow some analysis of past MHC haplotype frequencies and their comparison to modern patterns, in tandem with a neutral site may provide indirect evidence of a past epidemic. An understanding of past events may help in predicting future ones.

6.3.4 This technique could be used to investigate if the kiwi uses MHC based disassortive mating. It is understood kiwi mate choice is currently being investigated by some other researchers. If it does affect mate choice, then it will be an important factor in kiwi breeding and translocation decisions.

6.3.6 The MHC in other ratites may be amplified using the primers designed for kiwi. Work reported in Appendix E, although incomplete did indicate they may work in some of the more closely related ratites. Investigating the MHC in other ratites will illuminate how MHC evolved in Paleognathae. As emu and ostrich are farmed, it may allow more accessible investigation into MHC and disease response in ratites.

6.3.7 The use of MHC polymorphism or any of the new techniques and technologies that are being developed in conservation medicine for threatened species like the kiwi should create better opportunities to help our conservation management. However it is likely to be only part of a continual development process of better problem definitions and use of multipronged approaches to make effective use of current resources for conservation of the *Apteryx* species. How well we can understand the complexities of host parasite interactions and disease epidemiology through the lens of a populations MHC polymorphism is a question that we are still in the process of answering.

6.3.8 Ancient DNA (aDNA) is usually retrieved as small fragments of DNA. The seeming concentration of the balancing polymorphism in the BS1 & BS2 areas of Class II B exon 2, may make it easier to concentrate analyse of polymorphism to this area in old kiwi bones and tissue. Primers designed specifically for this area from this data, are more likely to find a small aDNA fragment than primers designed for the larger whole PBR (exon 2)

Appendix A

Table A.1 shows a consensus alignment of NIB kiwi DNA from exon 1 to 3, assembled from contiguous overlapping sequences. This was assembled from a combination of cDNA and genomic DNA. It is compared to 10 other similar bird sequences from the NCBI database. The GenBank accession numbers are supplied below. All these avian MHC Class II B DNA sequences showed a high level of similarity at the same region of Intron 2 (~65-83bp). It was expected that exons 2 & 3 would be similar while the introns would not to be similar as different evolutionary forces act on each (Cereb et al 1997; Garrigan and Edwards 1999). A Kozak sequence (Kozak 1996, 1999) was identified as the start from the cDNA, and is highlighted in yellow. The putative Exons 1 ,2 & 3 sequences are highlighted in green. The region of Intron 2 that appears to be conserved, is highlighted in purple. Only areas of the sequences that are similar have their nucleotide sequences shown, other section have a " - " in place of the nucleotide sequence, so differences in the number of bp's between regions are removed to allow a better comparison (Satta et al 1998).

Table A.1

```
                                  Exon 1>>
                            Kozak seq.
Kiwi_exon_1-3               GCCATGGGGACTGGTTGCGTGCAGGGCGCTGGGAGGCGCTGGGCTGGAGCAGCGTTAGCAGTGCTGGCG
ApNIBDRB-1_exon_2           ------------------------------------------------------------------------
S._humboldti_DRB1           ------------------------------------------------------------------------
B._buteo_Bubu-DRB           ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB    ------------------------------------------------------------------------
H._coronatus_Haco-DRB       ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB     ------------------------------------------------------------------------
G._africanus_Gyaf-DRB       ------------------------------------------------------------------------
G._barbatus_Gypa-DRB        ------------------------------------------------------------------------
C._gallicus_Ciga-DRB        ------------------------------------------------------------------------
H._pennatus_Hipe-DRB        ------------------------------------------------------------------------
P._haliaetus_Paha-DRB       ------------------------------------------------------------------------
```

```
                                                                Intron 1>>
Kiwi_exon_1-3                GTGCTGGGAGCCCCCGGGGCTCATGGCAAGGAGACGACAGGTGAGCTGAGTCCCCCGGTGGGGGGATGG
ApNIBDRB-1_exon_2            ------------------------------------------------------------------------
S._humboldti_DRB1            ------------------------------------------------------------------------
B._buteo_Bubu-DRB            ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB     ------------------------------------------------------------------------
H._coronatus_Haco-DRB        ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB      ------------------------------------------------------------------------
G._africanus_Gyaf-DRB        ------------------------------------------------------------------------
G._barbatus_Gypa-DRB         ------------------------------------------------------------------------
C._gallicus_Ciga-DRB         ------------------------------------------------------------------------
H._pennatus_Hipe-DRB         ------------------------------------------------------------------------
P._haliaetus_Paha-DRB        ------------------------------------------------------------------------

Kiwi_exon_1-3                CGTCAGGGGTCAGATGGCACAGACTCCCCGTGGAGGTGCTGGGGTTCGGGAGAGCCCCCTGGTGCTCTC
ApNIBDRB-1_exon_2            ------------------------------------------------------------------------
S._humboldti_DRB1            ------------------------------------------------------------------------
B._buteo_Bubu-DRB            ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB     ------------------------------------------------------------------------
H._coronatus_Haco-DRB        ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB      ------------------------------------------------------------------------
G._africanus_Gyaf-DRB        ------------------------------------------------------------------------
G._barbatus_Gypa-DRB         ------------------------------------------------------------------------
C._gallicus_Ciga-DRB         ------------------------------------------------------------------------
H._pennatus_Hipe-DRB         ------------------------------------------------------------------------
P._haliaetus_Paha-DRB        ------------------------------------------------------------------------
```

```
Kiwi_exon_1-3            ATGTCTCCTGTCAGGTCCAGGTGTCTCCACGGGGCGAGACACAGTCAGTTTGGGATGGCTCTGAGGAGT
ApNIBDRB-1_exon_2        ------------------------------------------------------------------------
S._humboldti_DRB1        ------------------------------------------------------------------------
B._buteo_Bubu-DRB        ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB ------------------------------------------------------------------------
H._coronatus_Haco-DRB    ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB  ------------------------------------------------------------------------
G._africanus_Gyaf-DRB    ------------------------------------------------------------------------
G._barbatus_Gypa-DRB     ------------------------------------------------------------------------
C._gallicus_Ciga-DRB     ------------------------------------------------------------------------
H._pennatus_Hipe-DRB     ------------------------------------------------------------------------
P._haliaetus_Paha-DRB    ------------------------------------------------------------------------

Kiwi_exon_1-3            CCCCGTGCCCTCCCCAACCTTGGGGAGCTGCACTCGACCCCCCCAACCTGCCTGAGGGCTGAGGGTGCC
ApNIBDRB-1_exon_2        ------------------------------------------------------------------------
S._humboldti_DRB1        ------------------------------------------------------------------------
B._buteo_Bubu-DRB        ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB ------------------------------------------------------------------------
H._coronatus_Haco-DRB    ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB  ------------------------------------------------------------------------
G._africanus_Gyaf-DRB    ------------------------------------------------------------------------
G._barbatus_Gypa-DRB     ------------------------------------------------------------------------
C._gallicus_Ciga-DRB     ------------------------------------------------------------------------
H._pennatus_Hipe-DRB     ------------------------------------------------------------------------
P._haliaetus_Paha-DRB    ------------------------------------------------------------------------
```

```
                                                                       (alternative initiation code -ctg ) Exon 2>>
Kiwi_exon_1-3                 TGGGGAGGAGGATGAGCTGGGGCACCGTGACCTGCCTCTCTCTGTACAACAGGGTATTTCCTGGAGATG
ApNIBDRB-1_exon_2             ------------------------------------------------------------CTGGCGATG
S._humboldti_DRB1             ----------------------------------------------------GGTATTTCCAGGAGATG
B._buteo_Bubu-DRB             -----------------------------------------------AACAGGGTTCTTCCAGGAGATG
N._percnopterus_Nepe-DRB      -----------------------------------------------AACAGGGTTTTTCCAGGAGATG
H._coronatus_Haco-DRB         ------------------------------------------------ACAGGGTTTTTCCAGGAGATG
G._coprotheres_Gyco-DRB       -----------------------------------------------AACAGGGTTTTTCCAGGAGATG
G._africanus_Gyaf-DRB         -----------------------------------------------AACAGGGTTTTTCCAGGAGATG
G._barbatus_Gypa-DRB          -----------------------------------------------AACAGGGTTTTTCCAGGAGATG
C._gallicus_Ciga-DRB          -----------------------------------------------AACAGGGTTTTTCCAGGAGATG
H._pennatus_Hipe-DRB          ---------------------------------------------------GGGTTTTTCCAGGAGATG
P._haliaetus_Paha-DRB         -----------------------------------------------AACAGGGTTTTTCCAGGAGATG


Kiwi_exon_1-3                 CATGCGTCCGAGTGTCAGTACCTCAACGGCACCGAGCAGGTGAGGTATTTGGATAGATACATCTACAAC
ApNIBDRB-1_exon_2             CATAAGGAAGAGTGTCAGTACCTCAATGGCACCGAGCGGGTCAGGTTGTTGGATAGATACATCTACAAC
S._humboldti_DRB1             GGTAAGGCCGAGTGTCATTTCCTCAACGGCACCGAGCGGGTGAGGTTTGTGGAGAGGTACATCTACAAC
B._buteo_Bubu-DRB             AGTAAGTCTGAGTGTCACCACCTCAATGGCAATAAGAATGTCAGGTATCTGGAGAAGTACATCTACAAC
N._percnopterus_Nepe-DRB      TTTAAGGCTGAGTGTCAGTACCTCAATGGCACAAAGCAGGTGAAGTATCTGGTCAAGTACATCTACAAC
H._coronatus_Haco-DRB         CATAAGTTTGAGTGTCACTACCTCAATGGCAACAAGAATGTGAGGTATCTGGAGAAGTACATCTACAAC
G._coprotheres_Gyco-DRB       AGTAAGTTTGAGTGTCAGTACCTCAATGGCAACAAGAATGTGAGGTATTTGCACAAGGACATCTACAAC
G._africanus_Gyaf-DRB         CATAAGTTTGAGTGTCAGTACCTCAATGGCAACAAGAACGTGAGGTTTCTCGACAAGTACATCTACAAC
G._barbatus_Gypa-DRB          TTTAAGGCTGAGTGTCAGTACCTCGATGGCACCAAGAATGTCAAGTTTCTGGATAAGTACGTCTACAAC
C._gallicus_Ciga-DRB          GGTAAGGGTGAGTGTCACTACCTCAATGGCAACAAGAATGTGAGGTATTTGCGCAAGGACATCTACAAC
H._pennatus_Hipe-DRB          GATAAGTTTGAGTGTCACTACCTCAATGGCAACAAGAATGTGAGGCTTTTGCACAAGAACATCTACAAC
P._haliaetus_Paha-DRB         GGCAAGAGTGAGTGTCAGTACCTCAATGGCAACAAGAACGTGAGGTTTCTGCACAAGTACATCTACAAC
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Kiwi_exon_1-3                CAGCAGCAGTACGTGCACTTCGACAGTGACGTGGGGTTCTACGTGGCCGACAGCCCCCTGGGTGAGCCA
ApNIBDRB-1_exon_2            CGGCAGCAGATCGTGCACTTCGACAGCGACGTGGGGTTCTACGTGGCCGACATCCCCCTGGTTGAACCA
S._humboldti_DRB1            CGGCAGCAGAACGTGCACTTCGACAGCGACGTGGGGTACTATGTGGCCGACACCCCCCTGGGTGAGCCT
B._buteo_Bubu-DRB            CGGGAGCAGAGGGTGCACTTTGACAGCGATGTGGGTCACTATGTGGCTGACACCCCCCTGGGTGAGCCT
N._percnopterus_Nepe-DRB     CGGGAGCAGAGGGCGCACTTTGACAGCGATGTGGGTCACTTTGTGGCCGACACCCCCCTGGGTGAGCCT
H._coronatus_Haco-DRB        CGGGAGCAGACTGTGCACTTTGACAGCGATGTGGGTCACTATGTGGCCGACACCCCCCTGGGTGAGCCT
G._coprotheres_Gyco-DRB      CGGGAGCAGACGGCACACTTTGACAGCGATGTGGGTCACTATGTGGCTGACACCCCCCTGGGTGAGCCT
G._africanus_Gyaf-DRB        CGGGAGCAGAGGGCACACTTTGACAGCGATGTGGGTCACTTTGTGGCCGACACCCCCCTGGGTGAGCCT
G._barbatus_Gypa-DRB         CGGGAACAGAGGGTGCACTTTGACAGCGATGTGGGTCACTTTGTGGCTGACACCCCCCTGGGTGAGCCT
C._gallicus_Ciga-DRB         CGGGAGCAGTTCGTGCACTTTGACAGCGATGTGGGTCACTTTGTGGCCGACAGCCCCCTGGGTGAGCCT
H._pennatus_Hipe-DRB         CGGGAGCAGATGTTGCACTTTGACAGCGATGTGGGTCACTATGTGGCCGACACCCCCCTGGGTGAGCCT
P._haliaetus_Paha-DRB        CGGGAGCAGAGAGCGCACTTTGACAGCGATGTGGGTCGCTTTGTGGCCGACACCCCCCTGGGTGAGCCT

Kiwi_exon_1-3                GATGCCAAGTACTGGAACAGTCAGCCAGACGTCATTGAGGAGGAACGGGCATCCGTGGACACGTACTGC
ApNIBDRB-1_exon_2            GATGCCAAGTACTGGAACGGCCAGCCAGACATCATTGAGCAGAAGCAGGCTGAGGTGGACACAGTGTGC
S._humboldti_DRB1            GATGCCAAGTACTGGAACAGCCAGACGGACTTCCTGGAGCAGAAACGGGCTGAGGTGGACACGTACTGC
B._buteo_Bubu-DRB            AGTGCCAAGTACTGGAACAGCCAACCGGACATACTGGAGGATGCACAGGCTGCGGTGGACACATACTGC
N._percnopterus_Nepe-DRB     AGTGCCAAGTACTGGAACAGCCTGCCGGAGGAACTGGAGTACAGACGGACCGCGGTGGACACGTTCTGC
H._coronatus_Haco-DRB        GATGCCAAGTACTGGAACAGCCAGCCGGACCTACTGGAGGATAAACAGGCTGACGTGGACACATTCTGC
G._coprotheres_Gyco-DRB      GATGCCAAGTACTGGAACAGCCAGCCGGACGTACTGGAGCGCAAACGGGCTGAGGTGGACGTGTGCCGA
G._africanus_Gyaf-DRB        TCTGCCAAGTACTGGAACAGCCAGCCCGACCTACTGGAGACCAGACGGGCTGCGGTGGACAGCTTCTGC
G._barbatus_Gypa-DRB         GATGCCAAGTACTGGAACAGCCAGCCGGACATACTAGAGGATGAACGGACTGCGGTGGACACATTCTGC
C._gallicus_Ciga-DRB         ACTGCCAAGTACTGGAACAGCCAGCCGGACATACTGGACAACACACGGGCTATGGTGGACACGTCCTGC
H._pennatus_Hipe-DRB         GATGCCAAGTACTGGAACAGCCAGCCGGACATACTGGAGAGGAAACAGGCTGAGGTTGACAGCGTCTGC
P._haliaetus_Paha-DRB        GATGCCAAGTACTGGAACAGCCAGCCGGACTTACTGGAGGAGGAACAGGCTGCGGTGGACAGGTTCTGC
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                                                                                                  Intron 2>>
Kiwi_exon_1-3                 CGGCACAACTATGGGGTGGCGACCCCTTTCACCGTGGAGAGGAGAGGTGAGTGAATGGGAGAGCCCTGG
ApNIBDRB-1_exon_2             CGGCACAACTATGGGGTGGGGACCCCTTTCACCGTGGAGAGGAGAGGT--------------------
S._humboldti_DRB1             CGACACAACTACGGGGTGGGGACCCCTTTCACTGTGGAGAGGAGAGGTGAGTG---------------
B._buteo_Bubu-DRB             CGCCACAACTACGAGGTGTTCAGACCTTTCACCGTGGAGAGGAGAGGTGAGT----------------
N._percnopterus_Nepe-DRB      CGACACAACTACGAGGTGTCGACCCCTTTCCTCGTGGAGAGGAGAGGTGAGT----------------
H._coronatus_Haco-DRB         CGACACAACTACGAGGTGAACAGCCCTTTCACTGTGGAGAGGAGAGGTGAGT----------------
G._coprotheres_Gyco-DRB       CACAACTACGAGGTGGTG---ACCCCTTTCCTCGTGGAGAGGAGAGGTGAGT----------------
G._africanus_Gyaf-DRB         CGACACAACTACAAGGGGGTGACCCCTTTCACCGTGGAGAGGAGAGGTGAGT----------------
G._barbatus_Gypa-DRB          CGACACAACTACGAGGTGGCGACCCCTTTCACCGTGCAGAGGAGAGGTGAGT----------------
C._gallicus_Ciga-DRB          CGACACAACTACGAGGTGTTGACCCCTTTCATCACAGAGAGGAGAGGTGAGT----------------
H._pennatus_Hipe-DRB          CGACTCAACTACGAGGTGTTCACCCCTTTCACCGTGGAGAGGAGAGGTGAGT----------------
P._haliaetus_Paha-DRB         CGACACAACTACGAGGCGGCGACCCCCTTCATCACAGAGAGGAGAGGTGAGT----------------


Kiwi_exon_1-3                 GTCTGGCGGGGCTGGGACACCCCCACGCCCTCCACACTGCAGGCAGCCGAGTGGGGGGGGCACAGGGGG
ApNIBDRB-1_exon_2             ---------------------------------------------------------------------
S._humboldti_DRB1             ---------------------------------------------------------------------
B._buteo_Bubu-DRB             ---------------------------------------------------------------------
N._percnopterus_Nepe-DRB      ---------------------------------------------------------------------
H._coronatus_Haco-DRB         ---------------------------------------------------------------------
G._coprotheres_Gyco-DRB       ---------------------------------------------------------------------
G._africanus_Gyaf-DRB         ---------------------------------------------------------------------
G._barbatus_Gypa-DRB          ---------------------------------------------------------------------
C._gallicus_Ciga-DRB          ---------------------------------------------------------------------
H._pennatus_Hipe-DRB          ---------------------------------------------------------------------
P._haliaetus_Paha-DRB         ---------------------------------------------------------------------
```

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Kiwi_exon_1-3            CGTCCCCGGAGGATGGCGATGAACCAGGCTGCTGGGATTGTGGGAGGGGAGCGTCCGTACCCAGCCGTG
ApNIBDRB-1_exon_2        ------------------------------------------------------------------------
S._humboldti_DRB1        ------------------------------------------------------------------------
B._buteo_Bubu-DRB        ------------------------------------------------------------------------
N._percnopterus_Nepe-DRB ------------------------------------------------------------------------
H._coronatus_Haco-DRB    ------------------------------------------------------------------------
G._coprotheres_Gyco-DRB  ------------------------------------------------------------------------
G._africanus_Gyaf-DRB    ------------------------------------------------------------------------
G._barbatus_Gypa-DRB     ------------------------------------------------------------------------
C._gallicus_Ciga-DRB     ------------------------------------------------------------------------
H._pennatus_Hipe-DRB     ------------------------------------------------------------------------
P._haliaetus_Paha-DRB    ------------------------------------------------------------------------
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Intron 2 area of high similarity>>

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Kiwi_exon_1-3            CCCACTGCAGGGGCCCGGGCGAGGTCCCTGCACCCTTCCACGAGGACACGAGCCCCTTGCGTCCTCCCT
ApNIBDRB-1_exon_2        ------------------------------------------------------------------------
S._humboldti_DRB1        -------CAGGGCCCCGGGCGG--CCCCCGCGCCCTTCCACGAGGACGCGAGTCCCCTGCAGCCTCCCT
B._buteo_Bubu-DRB        -------------------------CCTGCGCCCTTCCACGAGGACGCGAGTCCCTCGTACCCTCGCT
N._percnopterus_Nepe-DRB -------------------------CTTGCGCCCTTCCACGAGGACGCGAGTCCCTTGCCCCCTCGCT
H._coronatus_Haco-DRB    -------------------------CCTGCGCCCTTCCACGAGGACGCAAGTCCCTCGCCACCTCGCT
G._coprotheres_Gyco-DRB  -------------------------CCTGTGCCCTTCCATGAGGACGCGAGTCCCTTGCCCCCTCGCT
G._africanus_Gyaf-DRB    -------------------------CCTGCGCCCTTCCATGAGGACGCGAGTCCCTTGCCCCCTCGCT
G._barbatus_Gypa-DRB     -------------------------CCTGCGCCCTTCCACGAGGACGCGAGTCCCTCGCCCCCTTGCT
C._gallicus_Ciga-DRB     -------------------------CCTGCGCCCTTCCACGAGGACGCGAGTCCCTCGCCCCTTCGCT
H._pennatus_Hipe-DRB     -------------------------CCTGCGCCCTTCCACGAGGACGCGAGTCCCTCGCCCCCTCGCC
P._haliaetus_Paha-DRB    -------------------------CCTGCGCCCTTTCACAAGGACCCGAGTCCCTCGCCCCCTCGCC
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Kiwi_exon_1-3              GGACACATCTTGCGTGGGGACCACAACACACAGCCCTGGCCGGGATGAGTCCTGAGAGCAGCAGTGCCA
ApNIBDRB-1_exon_2          ------------------------------------------------------------------
S._humboldti_DRB1          GGGCACGTCCCGCGTGGGGACC---------------------------------------------
B._buteo_Bubu-DRB          GGGCACGTCCTGCGTGGGGACC---------------------------------------------
N._percnopterus_Nepe-DRB   GGGCACGTCCCACGTGGGGACC---------------------------------------------
H._coronatus_Haco-DRB      GGGCACGTCCTGCGTGGG-ACC---------------------------------------------
G._coprotheres_Gyco-DRB    GGGCACGTCCTGCGTGGGGACC---------------------------------------------
G._africanus_Gyaf-DRB      GGGCACGTCCTGCGTGGGGACC---------------------------------------------
G._barbatus_Gypa-DRB       GGGCACGTCCTGCGTGGGGACC---------------------------------------------
C._gallicus_Ciga-DRB       GGGCACGTCCTGCGTGGGGACC---------------------------------------------
H._pennatus_Hipe-DRB       GGGCACGTCCTGCGTGGGGACC---------------------------------------------
P._haliaetus_Paha-DRB      GGGCACGTCCTGCGTGGGGACC---------------------------------------------

                                                          (alternative initiation code -ctg )  Exon 3
Kiwi_exon_1-3              GGTGGCCCCTCACGCTCTCCCAGCTAATTCCAGCTCTCTCGCTCTCCCACAGTTCAGCCCAAGGTGCTG
ApNIBDRB-1_exon_2          ------------------------------------------------------------------
S._humboldti_DRB1          ----------------------------------------CCC-CAGTTCAGCCCAAGGTGAGG
B._buteo_Bubu-DRB          --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
N._percnopterus_Nepe-DRB   --------------------------------CTCTCTCGCTCTCCCCCAGTTCAGCCCAAGGTGAGG
H._coronatus_Haco-DRB      --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
G._coprotheres_Gyco-DRB    --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
G._africanus_Gyaf-DRB      --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
G._barbatus_Gypa-DRB       --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
C._gallicus_Ciga-DRB       --------------------------------CTCTCTCGCTCTCCCCCAGTTGAGCCCAAGGTGAGG
H._pennatus_Hipe-DRB       --------------------------------CTGTCTCGCTCTCCCGCAGTTGAGCCCAAGGTGAGG
P._haliaetus_Paha-DRB      --------------------------------CTCTCTTGCTCTCCCCCAGTTCAGCCCAAGGTGAGG
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Kiwi_exon_1-3              GTGTCCCCCATGCAGTCGGGGTCCCTGCCCCAAACGGACAGGCTGCTCTGCTACGTGACGGGCTTTTAC
ApNIBDRB-1_exon_2          ------------------------------------------------------------------------
S._humboldti_DRB1          GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCGACAGGCTGGTTTGCTACGTGACGGGCTTCTAC
B._buteo_Bubu-DRB          GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
N._percnopterus_Nepe-DRB   GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
H._coronatus_Haco-DRB      GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
G._coprotheres_Gyco-DRB    GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
G._africanus_Gyaf-DRB      GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
G._barbatus_Gypa-DRB       GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
C._gallicus_Ciga-DRB       GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
H._pennatus_Hipe-DRB       GTCTCCCCCATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTTTAC
P._haliaetus_Paha-DRB      GTCTCCCCAATGCAGTCGAGCTCCCTGCCCCAGACCAACAGGCTGGTTTGCTACGTGACGGGGTTCTAC

Kiwi_exon_1-3              CCGGCGGAGATCAAGGTCAAGTGGTTCAAGAACGGGCGGGAGGAGACGGAGCGCGTGGTGGCCACGGAC
ApNIBDRB-1_exon_2          ------------------------------------------------------------------------
S._humboldti_DRB1          CCCGCGGAGATCGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCGCGTGGTGTCCACGGAT
B._buteo_Bubu-DRB          CCCGCGGAGATCGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
N._percnopterus_Nepe-DRB   CCCGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
H._coronatus_Haco-DRB      CCCGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
G._coprotheres_Gyco-DRB    CCTGCGGAGATTGAGGTGAAGTGGTTCAGGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
G._africanus_Gyaf-DRB      CCCGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
G._barbatus_Gypa-DRB       CCCGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
C._gallicus_Ciga-DRB       CCCGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
H._pennatus_Hipe-DRB       CCTGCGGAGATTGAGGTGAAGTGGTTCAAGAACGGGCAGGAGGAGACGGAGCACGTGGTGTCCACGGAC
P._haliaetus_Paha-DRB      CCCGCGGAGATTGAGGTGAAGTGGTTCACGAACGGGCAGGAGGAGACGGAGCGCGTGGTGTCCACGGAC
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Kiwi_exon_1-3              GTGATCCAGAACGGCGACT
ApNIBDRB-1_exon_2          --------------------
S._humboldti_DRB1          GTGATCCAGAACGGAGACT
B._buteo_Bubu-DRB          GTGATCCAGAACGGAGACT
N._percnopterus_Nepe-DRB   ATGATCCAGAACGGAGACT
H._coronatus_Haco-DRB      ATGATCCAGAACGGAGACT
G._coprotheres_Gyco-DRB    ATGATCCAGAACGGAGACT
G._africanus_Gyaf-DRB      ATGATCCAGAACGGAGACT
G._barbatus_Gypa-DRB       ATGATCCAGAACGGAGACT
C._gallicus_Ciga-DRB       ATGATCCAGAACGGAGACT
H._pennatus_Hipe-DRB       ATGATCCAGAACGGAGACT
P._haliaetus_Paha-DRB      ATGATCCAGAACAGAGACT
```

Table A.2 The Bird sequences used to align with the kiwi exon 1-3 and their similarity at 3 areas.

Bird species	GenBank Accession No	similarity exon 2	similarity intron 2 piece.	similarity exon 3
Spheniscus humboldti	AB154398.1	87%	83%	89%
Bubo bubo	EF370956	78%	83%	88%
Neophron percnopterus	EF370964	79%	81%	88%
Harpyhaliaetus coronatus	EF370961	81%	83%	87%
Gypus coprotheres	EF370978	79%	83%	87%
Gypus africanus	EF370973	79%	84%	86%
Gypaetus barbatus	EF370960	81%	83%	87%
Ciraetus gallicus	EF370957	78%	83%	87%
Hieraaetus pennatus	EF370962	79%	83%	87%
Pandion haliaetus	EF370965	79%	80%	86%

The level of similarity to the kiwi sequence was estimated using Pair-wise analysis of p distances was in Mega 4 (Tamura et al 2007)

Appendix B

Table B.1 The size and distribution of the putative LSK Class II B alleles.

Species	Allele name	size of sequence	size of exon 2	No. of ORF's	No. of birds with allele.
LSK	Apow 1	281	258	1	all 8
	Apow 2	281	264	1	all 8
	Apow 3	281	264	1	4
	Apow 4	284	267	2	7

Table B.2 The nucleotide sequences of the four putative Little Spotted Kiwi Class II B alleles. The sequences are in pairs, the longer sequence still has some of the introns attached at either end of the exon while the sequence with the "exon 2" has only exon 2 base pairs.

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Apow_001          --C AAC AGG GTA TTT CCA GGA GAT GAA TAA ATA TGA GTG TCA GTT CCT CAA CGG CAC CGA GCG GGT GAG  [ 69]
Apow_001_exon_2   --- --- --- --- --- --- --- -AT GAA TAA ATA TGA GTG TCA GTT CCT CAA CGG CAC CGA GCG GGT GAG  [ 69]
Apow_002          CAC AAC AGG GTA TTT CCT GGA GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG  [ 69]
Apow_002_exon_2   --- --- --- --- --- -CT GGA GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG  [ 69]
Apow_003          CAC AAC AGG GTA TTT CCT GGA CAT GAG TAT ATT CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG  [ 69]
Apow_003_exon_2   --- --- --- --- --- -CT GGA CAT GAG TAT ATT CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG  [ 69]
Apow_004          CAC AAC AGG GTA TTT CCT GGA CAT GGG TAA AAC CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT CAG  [ 69]
Apow_004_exon_2   --- --- --- --- --- -CT GGA CAT GGG TAA AAC CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT CAG  [ 69]

Apow_001          GTT GGT GCA TAG GAG GAT CTA CAA CCG GCA GCA GTT GCT GCA CTT TGA CAG CGA CGT GGG GTT CTA TGT  [138]
Apow_001_exon_2   GTT GGT GCA TAG GAG GAT CTA CAA CCG GCA GCA GTT GCT GCA CTT TGA CAG CGA CGT GGG GTT CTA TGT  [138]
Apow_002          GTT GTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG CGA CGT GGG GGT CTT CGT  [138]
Apow_002_exon_2   GTT GTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG CGA CGT GGG GGT CTT CGT  [138]
Apow_003          GTA TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG CGA CGT GGG GGT CTA CGT  [138]
Apow_003_exon_2   GTA TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG CGA CGT GGG GGT CTA CGT  [138]
Apow_004          GTT TTT GGA GAG GCA CGT CTA CAA CCG GCA GCA GTT GGT GCA CTT TGA CAG TGA CGT GGG GTT CTA CGT  [138]
Apow_004_exon_2   GTT TTT GGA GAG GCA CGT CTA CAA CCG GCA GCA GTT GGT GCA CTT TGA CAG TGA CGT GGG GTT CTA CGT  [138]
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Apow_001         GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GCA GAG  [207]
Apow_001_exon_2 GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GCA GAG  [207]
Apow_002         GGC CGA CAT CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CGT TGA GCA GAG  [207]
Apow_002_exon_2 GGC CGA CAT CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CGT TGA GCA GAG  [207]
Apow_003         GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC TGA GTA CTG GAA TGG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apow_003_exon_2 GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC TGA GTA CTG GAA TGG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apow_004         GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GAC AGA GGT ACT GGA GCA TGC  [207]
Apow_004_exon_2 GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GAC AGA GGT ACT GGA GCA TGC  [207]

Apow_001         ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGT GGG GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_001_exon_2 ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGT GGG GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_002         ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_002_exon_2 ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_003         ACG GGG TGC GGT GGA CAG GTT CTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_003_exon_2 ACG GGG TGC GGT GGA CAG GTT CTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apow_004         ACA GAA TGC TGT GGA CAT GTT CTG CCG GCT CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apow_004_exon_2 ACA GAA TGC TGT GGA CAT GTT CTG CCG GCT CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]

Apow_001         AGG TG- -- [284]
Apow_001_exon_2 AGG T-- -- [284]
Apow_002         AGG TG- -- [284]
Apow_002_exon_2 AGG T-- -- [284]
Apow_003         AGG TG- -- [284]
Apow_003_exon_2 AGG T-- -- [284]
Apow_004         GAC AGG TG [284]
Apow_004_exon_2 GAC AGG T- [284]
```

Appendix C

Table C.1 The size and distribution of the putative NIB Class II B alleles.

North Island Brown Kiwi	Allele Name	size of sequence	size of exon 2	No. of ORF's	No. of birds with sequence.
NIB	Apma 001	281	264	1	2
	Apma 002	284	267	2	3
	Apma 003	281	264	1	4
	Apma 004	284	267	2	2
	Apma 005	281	264	1	3
	Apma 006	281	264	1	4
	Apma 007	284	267	2	2
	Apma 008	284	267	2	2
	Apma 009	281	264	1	2
	Apma 010	284	267	2	2
	Apma 011	281	264	1	2
NIB pseudogenes	Ap NIB DRB 2	281	186	1	3
	Ap NIB DRB 8	281	129	1	3

Table C.2 The nucleotide sequences of the eleven putative North Island Brown Kiwi Class II B alleles and two putative pseudogenes. The sequences are in pairs, the longer sequence still has some of the introns attached at either end of the exon while the sequence with the "exon 2" has only exon 2 base pairs.

Apma_001	CAC AAC AGG GTA TTT CCT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_001_exon_2	--- --- --- --- --- -CT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_002	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA AGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_002_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA AGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_003	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT GAG	[69]
Apma_003_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT GAG	[69]
Apma_004	CCC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TAA GCG GGT GAG	[69]
Apma_004_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TAA GCG GGT GAG	[69]
Apma_005	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT GAG	[69]
Apma_005_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT GAG	[69]
Apma_006	CAC AAC AGG GTA TTT CCT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_006_exon_2	--- --- --- --- --- -CT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apma_007	TGC AAC AGC ATA TTT CCT GGA GAT GAA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GAG GGT GAG	[69]
Apma_007_exon_2	--- --- --- --- --- -CT GGA GAT GAA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GAG GGT GAG	[69]
Apma_008	CAC AAC AGG GTA TTT CCT GGC GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCA GGT GAG	[69]
Apma_008_exon_2	--- --- --- --- --- -CT GGC GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCA GGT GAG	[69]
Apma_009	CAC AAC AGG GTA TTT CCT GCA GAT GTT TAA GGC CGA GTG TCA GTT CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apma_009_exon_2	--- --- --- --- --- -CT GCA GAT GTT TAA GGC CGA GTG TCA GTT CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apma_010	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apma_010_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apma_011	CAC AAC AGG GTA TTT CCT GGA AAT GCA TAA GGG CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT CAG	[69]
Apma_011_exon_2	--- --- --- --- --- -CT GGA AAT GCA TAA GGG CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT CAG	[69]
Ap_NIB_DRB_2	CAC AAC AGT GTA TTT CCT GGC GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCA GGT GAG	[69]
Ap_NIB_DBR_8	CAC AAC AGG GTA TTT CCT GGA GAT GGT TAA GGC CGA GTG TCA GTA TGT CAA CGG CAC CGA GCG GGT GAG	[69]

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Apma_001          GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_001_exon_2   GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_002          GTT GTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_002_exon_2   GTT GTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_003          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GCT GCA CTT CGA TAG CGA CGT GGG GGT CTA CGC  [138]
Apma_003_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GCT GCA CTT CGA TAG CGA CGT GGG GGT CTA CGC  [138]
Apma_004          GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_004_exon_2   GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_005          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GCT GCA CTT CGA TAG CGA CGT GGG GGT CTA CGC  [138]
Apma_005_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GCT GCA CTT CGA TAG CGA CGT GGG GGT CTA CGC  [138]
Apma_006          GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_006_exon_2   GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_007          GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG CGA CGT GGG GGT CTA TGT  [138]
Apma_007_exon_2   GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG CGA CGT GGG GGT CTA TGT  [138]
Apma_008          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_008_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT GGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apma_009          GTA TGT GGC TAG GAG CAT CTA CAA CCG GCA GCA GGA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_009_exon_2   GTA TGT GGC TAG GAG CAT CTA CAA CCG GCA GCA GGA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_010          GTT GGT GCA GAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAG CGA CGT GGG GGT CTA TGT  [138]
Apma_010_exon_2   GTT GGT GCA GAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAG CGA CGT GGG GGT CTA TGT  [138]
Apma_011          GTT GTT GGA GAG ATA CAT CTA CAA CCG GCA GCA GTA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apma_011_exon_2   GTT GTT GGA GAG ATA CAT CTA CAA CCG GCA GCA GTA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Ap_NIB_DRB_2      GTT TTT GGA TAG ATA GAT CCA CAA CCG GCA GCA GTT GGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Ap_NIB_DBR_8      GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCC ATT GCT GCA CTT CGA CAG CGA CGT AGG GTT CTA CAT  [138]
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Apma_001          GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAA  [207]
Apma_001_exon_2   GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAA  [207]
Apma_002          GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CAT TGA GCG GAA  [207]
Apma_002_exon_2   GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CAT TGA GCG GAA  [207]
Apma_003          GGC CGA CAG CCC CCT GGG TGA ACC ACT GGC CAA GGC CTG GAA CAG CCA GCC AGA GAT ACT GGA GCG TGC  [207]
Apma_003_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC ACT GGC CAA GGC CTG GAA CAG CCA GCC AGA GAT ACT GGA GCG TGC  [207]
Apma_004          GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apma_004_exon_2   GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apma_005          GGC CGA CAG CCC CCT GGG TGA ACC ACT GGC CAA GGC CTG GAA CAG CCA GCC AGA GAT ACT GGA GCG TGC  [207]
Apma_005_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC ACT GGC CAA GGC CTG GAA CAG CCA GCC AGA GAT ACT GGA GCG TGC  [207]
Apma_006          GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAA  [207]
Apma_006_exon_2   GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAA  [207]
Apma_007          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAA  [207]
Apma_007_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAA  [207]
Apma_008          GGC CGA CAT CCC CCT GGG TGA ACC AGA TGC CGA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apma_008_exon_2   GGC CGA CAT CCC CCT GGG TGA ACC AGA TGC CGA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apma_009          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apma_009_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apma_010          GGC CAA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA CAG  [207]
Apma_010_exon_2   GGC CAA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA CAG  [207]
Apma_011          GGC TGA CAG CCC CCT GGG TGA GCC AAG TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA CAG  [207]
Apma_011_exon_2   GGC TGA CAG CCC CCT GGG TGA GCC AAG TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA CAG  [207]
Ap_NIB_DRB_2      GGC CGA CAT CCC CCT GGG TGA ACC AGA TGC CGA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Ap_NIB_DBR_8      GGC CGA CAG CCC CCT AGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCT GAC AGA CTT CAT TGA GCG GGA  [207]
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Apma_001          GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGG GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_001_exon_2   GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGG GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_002          ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA TCA TGT GGT TGG CAG  [276]
Apma_002_exon_2   ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA TCA TGT GGT TGG CAG  [276]
Apma_003          ACA GAA TGC TGT GGA CAC GAT CTC CCG GCA CAA CTC TTG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_003_exon_2   ACA GAA TGC TGT GGA CAC GAT CTC CCG GCA CAA CTC TTG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_004          ACA GAA TGC TCT GGA CAT GTT CTG CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_004_exon_2   ACA GAA TGC TCT GGA CAT GTT CTG CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_005          ACA GAA TGC CAT GGA CAC GAT CTC CCG GCA CAA CTA TTG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_005_exon_2   ACA GAA TGC CAT GGA CAC GAT CTC CCG GCA CAA CTA TTG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_006          GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_006_exon_2   GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_007          ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CCG  [276]
Apma_007_exon_2   ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CCG  [276]
Apma_008          ACG GGC GTC TGA GAA CAG GTT CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_008_exon_2   ACG GGC GTC TGA GAA CAG GTT CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_009          ACG GGC CAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_009_exon_2   ACG GGC CAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_010          ACG GGC TGA GGT GGA CAC GTT CTG CCG GTG CAG CTA TGT GGT GGT TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_010_exon_2   ACG GGC TGA GGT GGA CAC GTT CTG CCG GTG CAG CTA TGT GGT GGT TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apma_011          ACG GGC TGC AGT GGA CAC GTT CTG CCG GCA CAA CTA TGT GGG GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apma_011_exon_2   ACG GGC TGC AGT GGA CAC GTT CTG CCG GCA CAA CTA TGT GGG GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Ap_NIB_DRB_2      ACG GGC GTC TGA GAA CAG GTT CTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Ap_NIB_DBR_8      ACA GGG TGA GGT GGA CAG GTT GTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CGT GGA GAG GAG  [276]
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Apma_001          AGG TG- -- [284]
Apma_001_exon_2   AGG T-- -- [284]
Apma_002          GAC AGG TG [284]
Apma_002_exon_2   GAC AGG T- [284]
Apma_003          AGG TG- -- [284]
Apma_003_exon_2   AGG T-- -- [284]
Apma_004          GAC AGG TG [284]
Apma_004_exon_2   GAC AGG T- [284]
Apma_005          AGG TG- -- [284]
Apma_005_exon_2   AGG T-- -- [284]
Apma_006          AGG TG- -- [284]
Apma_006_exon_2   AGG T-- -- [284]
Apma_007          GAA AGG TG [284]
Apma_007_exon_2   GAA AGG T- [284]
Apma_008          GAG AGG TG [284]
Apma_008_exon_2   GAG AGG T- [284]
Apma_009          AGG TG- -- [284]
Apma_009_exon_2   AGG T-- -- [284]
Apma_010          GAC AGG TG [284]
Apma_010_exon_2   GAC AGG T- [284]
Apma_011          AGG TG- -- [284]
Apma_011_exon_2   AGG T-- -- [284]
Ap_NIB_DRB_2      AGG TG- -- [284]
Ap_NIB_DBR_8      AGG TG- -- [284]
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Appendix D

Table D.1 The size and distribution of the Rowi Class II B alleles.

Rowi	Allele name	size of sequence	size of exon 2	No of ORF's	No of birds with sequence.
Rowi	Apro 001	284	267	2	10
	Apro 002	284	267	2	4
	Apro 003	281	264	1	5
	Apro 004	284	267	2	3
	Apro 005	281	264	1	2
	Apro 006	281	264	1	4
	Apro 007	284	267	2	6
	Apro 008	281	264	1	6
	Apro 009	284	267	2	6
	Apro 010	281	264	1	5
	Apro 011	281	264	1	3
	Apro 012	284	267	2	2
	Apro 013	281	264	1	3
	Apro 014	281	264	1	2
Rowi pseudogenes	Ap ROW DRB 5	281	186	1	5
	Ap ROW DRB 6	283	103	1	2
	Ap ROW DRB 16	283	103	1	5

Table D.2 The nucleotide sequences of the fourteen putative Rowi Class II B alleles and three putative pseudogenes. The sequences are in pairs, the longer sequence still has some of the introns attached at either end of the exon while the sequence with the "exon 2" has only exon 2 base pairs.

Apro_001	TGC AAC AGC ATA TTT CCT GGA GGT GAA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GAG GGT GAG	[69]
Apro_001_exon_2	--- --- --- --- --- -CT GGA GGT GAA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GAG GGT GAG	[69]
Apro_002	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT CAG	[69]
Apro_002_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC TGA GCG GGT CAG	[69]
Apro_003	CAC AAC AGG GTA TTT CCT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apro_003_exon_2	--- --- --- --- --- -CT GGC GAT GCA TAA GGA AGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apro_004	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_004_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_005	CAC AAC AGG GTA TTT CCT GGA GAT GTT TAA GTT CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_005_exon_2	--- --- --- --- --- -CT GGA GAT GTT TAA GTT CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_006	CAC AAC AGG GTA TTT CCT GGC GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCA GGT GAG	[69]
Apro_006_exon_2	--- --- --- --- --- -CT GGC GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCA GGT GAG	[69]
Apro_007	CAC AAC AGG GTA TTT CCT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_007_exon_2	--- --- --- --- --- -CT GGA GAT GGG TAA ATC CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Apro_008	CAC AAC AGG GTA TTT CCT GGA GAT GGA TAA ATT TGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG	[69]
Apro_008_exon_2	--- --- --- --- --- -CT GGA GAT GGA TAA ATT TGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG	[69]
Apro_009	TAC AAC AGG GTA TTT CCT GCA GAT GTT TAA GGC CGA GTG TCA GTT CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apro_009_exon_2	--- --- --- --- --- -CT GCA GAT GTT TAA GGC CGA GTG TCA GTT CCT CAA TGG CAC CGA GCA GGT GAG	[69]
Apro_010	CGC AAC AGG GTA TTT CCT GGA GAT GCA TAA GTC CGA GTG TCA GTA TGT CAA CGG CAC TGA GCA GGT GAA	[69]
Apro_010_exon_2	--- --- --- --- --- -CT GGA GAT GCA TAA GTC CGA GTG TCA GTA TGT CAA CGG CAC TGA GCA GGT GAA	[69]
Apro_011	CAC AAC AGG GTA TTT CCT GGA GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apro_0011_exon_2	--- --- --- --- --- -CT GGA GAT GCA TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT CAG	[69]
Apro_012	TAC AAC AGG GTA TTT CCT GGA GAT GCT TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT GAG	[69]
Apro_012_exon_2	--- --- --- --- --- -CT GGA GAT GCT TAA GGC CGA GTG TCA GTA CCT CAA TGG CAC TGA GCG GGT GAG	[69]
Apro_013	CAC AAC AGG GTA TTT CCT GGA AAT GCA TAA GTC CGA GTG TCA GTA TGT CAA TGG CAC TGA GCA GGT GAG	[69]
Apro_013_exon_2	--- --- --- --- --- -CT GGA AAT GCA TAA GTC CGA GTG TCA GTA TGT CAA TGG CAC TGA GCA GGT GAG	[69]
Apro_014	CAC AAC AGG GTA TTT CCT GGA GAT GGA TAA ATT TGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG	[69]
Apro_014_exon_2	--- --- --- --- --- -CT GGA GAT GGA TAA ATT TGA GTG TCA GTA CCT CAA TGG CAC CGA GCG GGT GAG	[69]
Ap_ROW_DRB_-_5	CAC AAC AGG GTA TTT CCA GGA GGT GCA TAG GGC CGA GTG TCA ATA CCT CAA CGG CAC CGA GCA GGT CAG	[69]
Ap_ROW_DRB_-_6	TGC AAC AGG GTA TTT CCT GGA TAT GGA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]
Ap_ROW_DRB_-_16	TGC AAC AGG GTA TTT CCT GGA TAT GGA TAA GGG CGA GTG TCA GTA CCT CAA CGG CAC CGA GCG GGT GAG	[69]

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Apro_001          GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG TGA TGT GGG GGT CTA TGT  [138]
Apro_001_exon_2   GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT CGA CAG TGA TGT GGG GGT CTA TGT  [138]
Apro_002          GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG TGA AGT GGG GGT CTT CGT  [138]
Apro_002_exon_2   GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG TGA AGT GGG GGT CTT CGT  [138]
Apro_003          GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apro_003_exon_2   GTT GTT GGA TAG ATA CAT CTA CAA CCG GCA GCA GAT CGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apro_004          GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT ACA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apro_004_exon_2   GTT TTT GGT GAG GTA CAT CCA CAA CCG GCA GCA GTT TGT ACA CTT CGA CAG TGA CGT GGG GGT CTT CGT  [138]
Apro_005          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT CGT GCA CTT CGA CAG TGA CGT GGG GTT CTA CGT  [138]
Apro_005_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT CGT GCA CTT CGA CAG TGA CGT GGG GTT CTA CGT  [138]
Apro_006          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apro_006_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT CGA CAG CGA CGT GGG GTT CTA CGT  [138]
Apro_007          GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTA TGT GCA CTT CGA CAG TGA CGT GGG GTT CTA CGT  [138]
Apro_007_exon_2   GTT TTT GGA TAG ATA CAT CCA CAA CCG GCA GCA GTA TGT GCA CTT CGA CAG TGA CGT GGG GTT CTA CGT  [138]
Apro_008          GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAA CGA CGT GGG GTT CTA TGT  [138]
Apro_008_exon_2   GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAA CGA CGT GGG GTT CTA TGT  [138]
Apro_009          GTA TGT GGC TAG GAG CAT CTA CAA CCG GCA GCA GGA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT TGT  [138]
Apro_009_exon_2   GTA TGT GGC TAG GAG CAT CTA CAA CCG GCA GCA GGA CGT GCA CTT CGA CAG TGA CGT GGG GGT CTT TGT  [138]
Apro_010          GTA TGT GGA GAG GTA CCT CTA CAA CCG GCA GCA GTA CGT GCA CTT TGA CTG TGA CCT GGG GGT CTA TGT  [138]
Apro_010_exon_2   GTA TGT GGA GAG GTA CCT CTA CAA CCG GCA GCA GTA CGT GCA CTT TGA CTG TGA CCT GGG GGT CTA TGT  [138]
Apro_011          GTT TTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG CGA CGT GGG GGT CTT CGT  [138]
Apro_0011_exon_2  GTT TTT GGA TAG ATA CAT CTA CAA CCA GCA GCA GTA CGT GCA CTT CGA CAG CGA CGT GGG GGT CTT CGT  [138]
Apro_012          GTT CGT GGT GAG GAA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT TGA CAG TGA TGT GGG GGT CTA CGT  [138]
Apro_012_exon_2   GTT CGT GGT GAG GAA CAT CCA CAA CCG GCA GCA GTT TGT GCA CTT TGA CAG TGA TGT GGG GGT CTA CGT  [138]
Apro_013          GTA TGT GGA GAG GTA CAT CCA CAA CCG GCA GCA GTT GGT GCA CTT TGA CTG TGA CCT GGG GGT CTA TGT  [138]
Apro_013_exon_2   GTA TGT GGA GAG GTA CAT CCA CAA CCG GCA GCA GTT GGT GCA CTT TGA CTG TGA CCT GGG GGT CTA TGT  [138]
Apro_014          GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAA CGA CGT GGG GTT CTA TGT  [138]
Apro_014_exon_2   GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAA CGA CGT GGG GTT CTA TGT  [138]
Ap_ROW_DRB_-_5    GTT TGT GGA GAG GTA CAT CCA CAA CTG GCA GCA GTT TGT GCA CTT CGA CAG CGA CGT GGG GGT CTT CGT  [138]
Ap_ROW_DRB_-_6    GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAG CGA CGT GGG GTC TAT GTG  [138]
Ap_ROW_DRB_-_16   GTT TGT GCA TAG GAA CAT CTA CAA CGG GCA GCT GAT GCT GCA CTT TGA CAG CGA CGT GGG GTC TAT GTG  [138]
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Apro_001          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAA  [207]
Apro_001_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAA  [207]
Apro_002          GGC CGA CAT CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_002_exon_2   GGC CGA CAT CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_003          GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GAC AGA CAT CAT TGA GCA GAA  [207]
Apro_003_exon_2   GGC CGA CAT CCC CCT GGT TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GAC AGA CAT CAT TGA GCA GAA  [207]
Apro_004          GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_004_exon_2   GGC CGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_005          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG TCA GCC AGA CCT CAT TGA GGA GGA  [207]
Apro_005_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG TCA GCC AGA CCT CAT TGA GGA GGA  [207]
Apro_006          GGC CGA CCT CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apro_006_exon_2   GGC CGA CCT CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apro_007          GGC CGA CAG CCC CCT GGG TGA GCC AAA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAG  [207]
Apro_007_exon_2   GGC CGA CAG CCC CCT GGG TGA GCC AAA TGC CAA GTA CTG GAA CAG CCA GCC AGA CTT CAT TGA GCA GAG  [207]
Apro_008          GGC TGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAG  [207]
Apro_008_exon_2   GGC TGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCA GCC AGA CAT CAT TGA GCA GAG  [207]
Apro_009          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apro_009_exon_2   GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAG  [207]
Apro_010          GGC TGA CAG TCC CCT GGG CGA GCC AAC TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apro_010_exon_2   GGC TGA CAG TCC CCT GGG CGA GCC AAC TGC CAA GTA CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA GAA  [207]
Apro_011          GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CGT TGA GCA GAG  [207]
Apro_0011_exon_2  GGC CGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CGG CCT GAC AGA CTT CGT TGA GCA GAG  [207]
Apro_012          GGC CGA CAG CCC CCT GGG TGA GCC AAC TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_012_exon_2   GGC CGA CAG CCC CCT GGG TGA GCC AAC TGC CAA GTA CTG GAA CAG CCA GCC AGA GGT ACT GGC GCA TGC  [207]
Apro_013          GGC TGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CAT CAT TGA GGA GAA  [207]
Apro_013_exon_2   GGC TGA CAG CCC CCT GGG TGA GCC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CAT CAT TGA GGA GAA  [207]
Apro_014          GGC TGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CAT CAT TGA GCA GAG  [207]
Apro_014_exon_2   GGC TGA CAG CCC CCT GGG TGA ACC AGA TGC CAA GTA CTG GAA CAG CCA GCC AGA CAT CAT TGA GCA GAG  [207]
Ap_ROW_DRB_-_5    GGC CGA CAT CCC CCT GGG TGA GCC AAG TGC CAA GTC CTG GAA CAG CCA GCC AGA CCT CAT TGA GGA CAG  [207]
Ap_ROW_DRB_-_6    GCT GAC AGC CCC CTG GGT GAA CCA GAT GCC GAG TAC TGG AAC AGC CAG CCA GAC CTC ATT GAG GAG AGA  [207]
Ap_ROW_DRB_-_16   GCT GAC AGC CCC CTG GGT GAA CCA GAT GCC GAG TAC TGG AAC AGC CAG CCA GAC CTC ATT GAG GAG AGA  [207]
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Apro_001          ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CCG  [276]
Apro_001_exon_2   ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CCG  [276]
Apro_002          ACA GAA TGC TCT GGA CAC GTT CTC CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TAG CAG  [276]
Apro_002_exon_2   ACA GAA TGC TCT GGA CAC GTT CTC CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TAG CAG  [276]
Apro_003          GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_003_exon_2   GCA GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_004          ACA GAA TGC TCT GGA CAC GTT CTG CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_004_exon_2   ACA GAA TGC TCT GGA CAC GTT CTG CCG GTG CAG CTA TGA GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_005          AAG GGC ATC CGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_005_exon_2   AAG GGC ATC CGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_006          ACG GGT GTC TGA GAA CAG GTT CTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_006_exon_2   ACG GGT GTC TGA GAA CAG GTT CTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_007          ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_007_exon_2   ACG GGG TGC GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_008          ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CAT GGA GAG GAG  [276]
Apro_008_exon_2   ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CAT GGA GAG GAG  [276]
Apro_009          ACG GGC CAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_009_exon_2   ACG GGC CAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG CAG  [276]
Apro_010          ACA GGC TAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_010_exon_2   ACA GGC TAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGT GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_011          ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_0011_exon_2  ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GGC GAC CCC TTT CAC CGT GGA GAG GAG  [276]
Apro_012          ACA GAA TGC TGT GGA CAC GTT CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG ACG  [276]
Apro_012_exon_2   ACA GAA TGC TGT GGA CAC GTT CTG CCG GCA CAA CTA TGG GGT GGC TCA AGC AGA CCA TGT GGT TGG ACG  [276]
Apro_013          ACG GGC TAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGA TGC CCC TTT CAC TGT GGA GAG GAG  [276]
Apro_013_exon_2   ACG GGC TAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GGA TGC CCC TTT CAC TGT GGA GAG GAG  [276]
Apro_014          ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CAT GGA GAG GAG  [276]
Apro_014_exon_2   ACG GGC TGA GGT GGA CAC AGT GTG CCG GCA CAA CTA TGG GGT GTT CTC CCC TTT CAC CAT GGA GAG GAG  [276]
Ap_ROW_DRB_-_5    ACG GGC TAA GGT GGA CAC GTA CTG CCG GCA CAA CTA TGG GGT GTT CAC CCC TTT CAC CGT GAA GAG GAA  [276]
Ap_ROW_DRB_-_6    CGG GGT GAG GTG GAC ACA CTC TGC CGG CAC AAC TAT GGG GTG GCT CAA GCA GAC CAT GTG GTT GGC CGG  [276]
Ap_ROW_DRB_-_16   CGG GGT GAG GTG GAC ACA CTC CGC CGG CAC AAC TAT GGG GTG GCT CAA GCA GAC CAT GTG GTT GGC CGG  [276]
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Apro_001          GAA AGG TG [284]
Apro_001_exon_2   GAA AGG T- [284]
Apro_002          GCC AGG TG [284]
Apro_002_exon_2   GCC AGG T- [284]
Apro_003          AGG TG- -- [284]
Apro_003_exon_2   AGG T-- -- [284]
Apro_004          GAC AGG TG [284]
Apro_004_exon_2   GAC AGG T- [284]
Apro_005          AGG TG- -- [284]
Apro_005_exon_2   AGG T-- -- [284]
Apro_006          AGG TG- -- [284]
Apro_006_exon_2   AGG T-- -- [284]
Apro_007          GAC AGG TG [284]
Apro_007_exon_2   GAC AGG T- [284]
Apro_008          AGG TG- -- [284]
Apro_008_exon_2   AGG T-- -- [284]
Apro_009          GAG AGG TG [284]
Apro_009_exon_2   GAG AGG T- [284]
Apro_010          AGG TG- -- [284]
Apro_010_exon_2   AGG T-- -- [284]
Apro_011          AGG TG- -- [284]
Apro_0011_exon_2  AGG T-- -- [284]
Apro_012          GAA AGG TG [284]
Apro_012_exon_2   GAA AGG T- [284]
Apro_013          AGG TG- -- [284]
Apro_013_exon_2   AGG T-- -- [284]
Apro_014          AGG TG- -- [284]
Apro_014_exon_2   AGG T-- -- [284]
Ap_ROW_DRB_-_5    AGG TG- -- [284]
Ap_ROW_DRB_-_6    AAA GGT G- [284]
Ap_ROW_DRB_-_16   AAA GGT G- [284]
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Appendix E

Application of KiwintA1F & KiwintA2R primers to other Paleognathae.

The primers KiwintA1F/KiwintA2R have been shown in this project to work in several different Kiwi species, so an attempt was made to see if they might work in other ratites. Only a limited range of samples were available for testing. First the samples were amplified with the KiwintA1F/KiwintA2R primers, then an aliquot of the PCR product was tested with the 325/326 primers which are internal to the KiwintA1F/KiwintA2R set. This was done to increase the likelihood that the PCR product produced was likely to be Class II MHC and not another section of DNA.

Method:

The PCR for primers KiwintA1F/KiwintA2R was performed in a 25µl volume containing PCR Reaction Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl,), 2.0 mM MgCl2 , 160µM dNTP, 0.8 mM of each primer, 1M Betaine, 0.5 units Platinum Taq (Invitrogen) and 1µl of DNA.. The PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/50°C for 30 seconds/72°C for 60 seconds for 30 cycles, then finished with a final 10 minute extension at 72°C. At this point the PCR product was checked using AGE (see chapter 3 section 3.4).

The second PCR used a 2µl aliquot from each PCR. The PCR Conditions for the primers 325 & 326 were performed in 25µl volumes containing Invitrogen PCR Reaction Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 2.5mM MgCl2 , 200µM dNTP, 0.5 units Invitrogen Platinum Taq, 1.6 µM of each primer. The PCR amplification was carried out in a GeneAmp 9600 Thermal Cycler as follows: after an initial step of 94°C for 2 minutes, the temperature was cycled 94°C for 30 seconds/50°C for 30 seconds/72°C for 30 seconds, for 20 cycles then finished with a final 30 second extension at 72°C. At this point the PCR product was checked using AGE (see chapter 3 section 3.4).

Results:

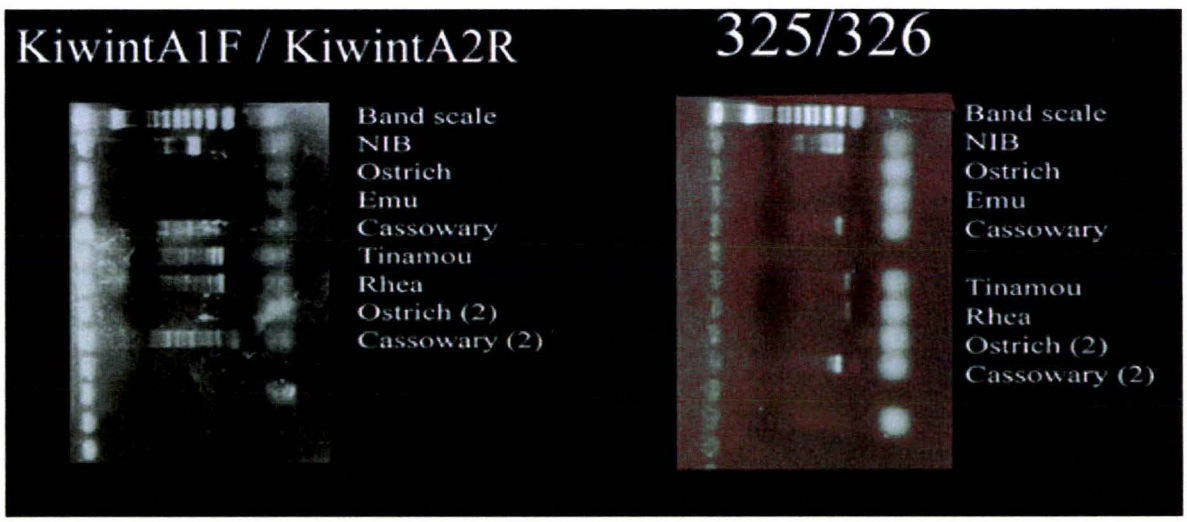

Figure E.1 The PCR bands produced by a NIB, Ostrich, Emu, Cassowary, Tinamou, and Rhea with KiwintA1F/KiwintA2R. This result shows no visible PCR product for Ostrich and Emu, but bands of product for Cassowary, Tinamou and Rhea. The NIB was used as a positive control, and a PCR mixture without DNA as the negative control. The picture on the right shows the PCR bands produced by the PCR product of NIB, Ostrich, Emu, Cassowary, Tinamou, and Rhea with KiwintA1F/KiwintA2R when used as the template for a second PCR with 325/326. This result suggests the PCR product found in the Cassowary, Tinamou and Rhea could well be from Class II B MHC.

Discussion:

These results suggest the KiwintA1F/KiwintA2R primers may work well in Cassowary, Tinamou and Rhea. but not in the Emu and Ostrich. This is only a preliminary result and more work up with a greater number of samples is required. A sequencing of the PCR product by a method similar to the one used in this project on Kiwi to minimise artefact formation would allow a more in-depth analysis of this result. These results are interesting as they are not consistent with the relationship pattern among ratites found by examination and comparison of mitochondrial DNA (Cooper et al 2001). As seen in Figure E.1 the emu and cassowary are more closely related than the rhea, so the appearance of the primers for *Apteryx* spp. working in rhea and tinamou (non ratite) is

unusual. This could be due to a problem with the quality and small number of samples used. Another possibility, was mentioned in chapter one, MHC can show loss and gain of various loci among species, and this has been seen in other species e.g. domestic cats, cattle and sheep(Yuhki et al 2003).

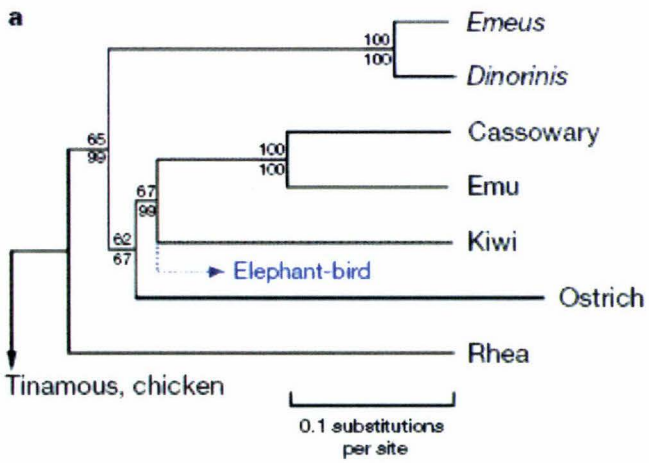

Figure E.2 Unconstrained maximum-likelihood tree of ratite taxa, generated by Cooper et al.(2001).

Appendix F

The Cup and Ball Model of selective forces on MHC

This model is my attempt to use a visual representation as a possible explanation to why the resulting selective force found at the molecular level operating on MHC is often weak. Like all models it is limited to the purpose for which it is made. It tries and connects a population's phenotypic response to pathogens with the resulting genetic pattern. However to do this it uses multiple assumptions and generalisation to reduce the model to 3 major interactions: The "cup", the "ball", and the force acting on the ball **Fpath.**

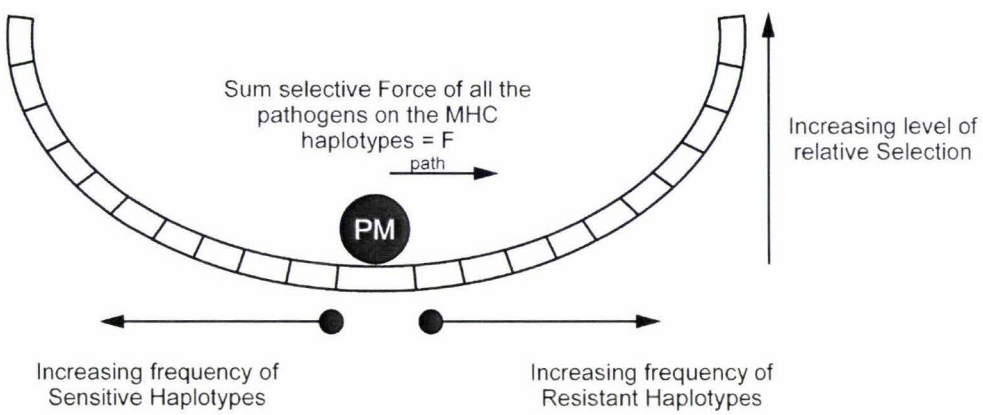

Figure F.1 The Cup and Ball Model of selective forces on MHC

Basic Assumptions:

My model assumes that the host MHC and pathogens are co-evolving; it is a dynamic relationship that varies over space and time. It is a complex relationship, with other intervening factors not the least of which is life history, modifying the selective force acting on the MHC of the host.

1. **Fpath.** The selection forces of the various pathogens acting on the host MHC, is not coherent in the wild but can be thought of as a variety of force vectors that can be summarised to a single resulting vector, **Fpath.** The **Fpath** sum force vector can only

be considered to act for a given instant on the host populations MHC. The individual force vector of each pathogen that the host population is exposed, is a function of such factors as the individual pathogens virulence and transmitablity. Just as the nature of the host population e.g., age structure & previous exposure, changes over time so does the mixture of pathogens change. This is why **Fpath** can be thought of as changing over time and space. **Fpath** also allows for pathogens to work in synergy or to inhibit one another which will change its strength and direction. A pathogens lifecycle can affect **F path** e.g., Malaria without a suitable mosquito vector is unable to be transmitted, hence it will not be transmitted to another in the population and its effects are limited to the carrier. However malaria with a vector and more than one host species has greater potential to spread even when one of the hosts is in small isolated populations, especially if the other host population is large and in the same area. The larger population acts as a reservoir of infection. The ultimate response to the **F path** by a host population is a change in the frequency of alleles, with more resistant MHC haplotypes types being more likely to be retained over more susceptible MHC haplotypes i.e. either a greater percentage of susceptibles are removed by dying or having fewer or no offspring compared to the more resistant. But by selecting for more resistance in the host population, a pathogen decreases the number of susceptible hosts available i.e., reducing its own force vector **F path**. As co-evolving host and parasite, the parasite has changed the allele frequency by selecting for more resistant haplotypes in the host and in turn over time the more resistant host can select more resistant parasites, changing both species haplotypes i.e., a dynamic interaction. If other factors such as disassortive mating are involved, then this will act to reduce **Fpath**, by weakening its overall selection force like a gravity tending to roll the ball to a different equilibrium. The interaction involves the host and a multiple of pathogens so the next time (instance) for which the **Fpath** is calculated the size and direction may have changed. Another pathogen may be the predominant influence hence the very topography of the "cup" with the tiles and the allele frequency they represent has changed. This makes a clear progression in a single direction i.e., a selective sweep favouring a single MHC genotype difficult to identify.

2. The "cup" is a relative 2D representation of possible haplotype frequencies in the population. The topography of the sides of the cup is made up of "tiles". Each tile

represents a different frequency of the MHC alleles in the population. Each tile has a "gradient" which represents the sum resistance or sensitivity to **F path** of all the alleles in proportion to their frequency i.e. the population's susceptibility at that allelic frequency. The size of tiles is in part a function of host generation intervals. If several haplotype frequencies in a population are equally resistant or susceptible then the topography of that region is flat. This use of a genetic topography is in part inspired by Sewell Wrights' adaptive topography (Wright 1931).

3. The ball is a marker of an instant in time. The size of the ball is a function of the polymorphism and the population size of the host. It is assumed a large population with allot of polymorphism is less susceptible to changing its allele frequency in this dynamic equilibrium e.g., a large polymorphic population would have a wide range of sensitivity/resistance responses not a limited on/off response being resistant or not resistant. The size of the ball is a visual indicator of the some of the forces interacting on **F path.**

This model allows for changes in either host or pathogen and therefore **F path** or arrangement and nature of the tiles can change e.g. an increased "stress" on the population like poor food supply can increase its susceptibility to infection which lowers the gradient of the tiles or may increase **F path.** Or a new pathogen e.g. emerging infectious disease changes **F path** and if the relative sensitivity or resistance of the Class I and II alleles is different then the ball can be thought of as going in a different direction in the 3D cup, but the topography i.e. gradient of each "tile" is different, as well as which alleles are resistant and sensitive, although the "ball" still moves to the right, showing an increasing frequency of resistant genes in the host population.

Example 1: In a MHC monomorphic population, or population where all the MHC haplotypes are equally effective/ineffective, the shape of the "cup" is flat – even if 99.9 % of the population is removed by an epidemic the genetic pattern of the MHC has not changed (beyond random changes and gene linkage interactions), the population is all equally resistant and no selection at the MHC has occurred and F_{path} is effectively = 0.

Certainly other forces like genetic drift or disassortive mating may still be acting on and affecting the resulting gene frequency.

Example 2: A pathogen is acting to create a **F path** on a PBR of the MHC. There are 10 alleles in the population, all at the same frequency 20%. So the initial instance tile in a population of 100 is 10a:10b:10c:10d:10e. The resistance is in increasing order i.e. A<B<C<D<E. The next instance due to **F path** the "ball" may be at the 19:20:20:20:20: tile. The tile next to it on the right might be 18:19:20:20:20 i.e. the population has not reproduced since the loss of susceptible members. It may progress to a new gene frequency like 1:5:9:20:25:35 as new generation appears, depending how the resistance has affected the various phenotypes fitness. At the next instance another factor may dominate and **F path** may act in another direction on another 'topography', resulting in another gene frequency pattern.

Appendix G

Table G.1 Table of MHC Class II exon 2 sequences used in NeighborNet analysis.

No	Bird name	NCBI Accession No	Abbrev. ID
1	*Aquila chrysaetos*	EF370905.1	Aqch1
2	*Pandion haliaetus*	EF370965.1	Paha1
3	*Elanus caeruleus*	EF370924.1	Elca1
4	*Elanus caeruleus (2)*	EF370925.1	Elca2
5	*Aegypius monachus*	EF370891.1	Aemo2
6	*Neophron percnopterus*	EF370964.1	Nepe 1
7	*Neophron percnopterus (2)*	EF370894.1	Nepe 2
8	*Aegypius monachus*	EF370954.1	Aemo1
9	*Gypus africanus*	EF370967.1	Gyaf2
10	*Gypus africanus (2)*	EF370968.1	Gyaf3
11	*Gypus coprotheres*	EF370976.1	Gyco1
12	*Gypus coprotheres (2)*	EF370978.1	Gyco2
13	*Ciraetus gallicus*	EF370913.1	Ciga1
14	*Ciraetus gallicus (2)*	EF370914.1	Ciga2
15	*Milus milvus*	EF370963.1	Mimil1
16	*Buteo buteo*	EF370899.1	Butbu1
19	*Buteo buteo (2)*	EF370956.1	Butbu2
17	*Harpyhaliaetus coronatus*	EF370961.1	Haco1
18	*Harpyhaliaetus coronatus (2)*	EF370902.1	Haco2
20	*Hieraaetus pennatus*	EF370909.1	Hipe1
21	*Hieraaetus pennatus (2)*	EF370910.1	Hipe2
22	*Aquila chrysaetos*	EF370955.1	Acqh2
23	*Circus aeruginosus*	EF370921.1	Ciae3
24	*Accipiter gentilis*	EF370953.1	Acge1
25	*Accipiter gentilis (2)*	EF370918.1	Acge2
26	*Asio otus*	EF370983.1	Asot1
27	*Asio otus (2)*	EF370940.1	Asot2
28	*Strix aluco*	EF370933.1	Stal1
29	*Strix aluco (2)*	EF370934.1	Stal2
30	*Athene noctua*	EF370942.1	Atno1
31	*Athene noctua (2)*	EF370943.1	Atno2
32	*Otus scops*	EF370981.1	Otsc1
33	*Otus scops (2)*	EF370938.1	Otsc2
34	*Bubo bubo*	EF370930.1	Bubbu2
35	*Bubo bubo (2)*	EF370931.1	Bubbu3
36	*Tyto alba*	EF370927.1	Tyal1
37	*Tyto alba (2)*	EF370979.1	Tyal2
38	*Falco biarmicus*	EF370989.1	Fabi1
39	*Falco biarmicus (2)*	EF370950.1	Fabi2
40	*Falco peregrinus*	EF370985.1	Fape1
41	*Falco peregrinus (2)*	EF370948.1	Fape2

42	*Falco femoralis*	EF370988.1	Fafe1
43	*Falco femoralis (2)*	EF370952.1	Fafe2
44	*Aphelocoma coerulescens*	U23975	Apco
45	*Spheniscus humboldti*	AB154398	
46	*Spheniscus humboldti (2)*	AB154393	
47	*Spheniscus mendiculus*	EF212007.1	Spme1
48	*Pygoscelis papua*	AB043597.1	Pypal.08
49	*Eudyptula minor*	AB060949	
50	*Phasianus colchicus*	X75407.1	
51	*Gallus gallus BLB2*	DQ885562	
52	*Gallus gallus BLB2 (2)*	AY770603	
53	*Corturnix mexicanus*	U23976	
54	*Agelaius phoeniceus*	U23970	
55	*Petroica traversi*	AY730418	
56	*Petroica australis australis*	AY730460	
57	*Gallinago media (snipe)*	AY694400.1	

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